SOME ASPECTS OF THE EFFECT OF GOSSYPOL ON ANTIFERTILITY

by

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ABSTRACT

Gossypol, a polyphenolic compound found in cotton plants, has a variety of possible pharmacologic uses. To explore more about its antifertility mechanism, the effect of gossypol on the LDH-X activity in cock and drake, the zinc metabolism in hamster and mouse and the antioxidant defense system in hamster were studied. This study also includes the effect of diet on gossypol efficacy.

Unlike Zinkham's finding, the LDH-X activity was evidently detected both in cock and drake. However, only the drake's testis was sensitive to gossypol. From a consideration of the enzyme level and its sensitivity to gossypol, it is interpreted that LDH-X may not be the target enzyme of gossypol unless an altered threshold is exhibited by cock.

Gossypol did not affect the zinc content in hair and testis of hamster and mouse. Yet, a marked increase in zinc content of these two animals was observed in their retina. We do not know the relationship between this change and that with the antifertility mechanism of gossypol.

On the other hand, some free radical scavengers such as vitamin C and selenium could eliminate the antifertility effect of gossypol. It was found that MDA (the product of lipid peroxidation) concentration in the testis of

gossypol-treated hamster was higher than that of the control. This effect bears a concentration relationship with the scavengers used. However, the gossypol-treated animals did not show a significant reduction pattern in the majority of antioxidant defense enzymes. On account of the fact that the total free radical in the system is a summation of many interrelated pathways, and that a definite change in the MDA level has been noted, it is plausible that the mechanism of gossypol might be related to the production of oxygen reactive species through metabolism.

At last, we also observed that lard could enhance the absorption and accumulation of gossypol. Then the antifertility effect of gossypol could be intensified by lard. Therefore, should gossypol be adopted to be used clinically, the effect of diet, especially that of fat, should be seriously considered.

CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

Nowadays, the rapid population growth is a major problem in the world. In many nations, particularly in the Third World, the exponential growth in population has hampered many social development and improvement of the quality of life. In this case, the birth rate must be controlled in order to pull the limited resources into other poorly developed aspects, such as education and industries. The Western countries have put a great deal of effort in birth control and family planning by means of various contraceptive methods. However, they are altogether not as effective as expected, except for sterilisation by surgery. The latter is not so well received by people , especially in the Chinese population as they thought that it may causes permanent infertility. Therefore, many countries are still searching for better and improved techniques for birth control.

Since the development of oral contraceptives for women, there have been efforts to identify suitable drugs for inhibition of male fertility. A variety of antimetabolic agents and steroid hormones are known to suppress the production of sperm by the testis. However, the antimetabolic agents cannot be seriously considered as potential contraceptives because of their general systemic toxicity.

At appropriate dose levels, the various hormones, including estrogens, progestins, androgens, and antiandrogens, block the production of sperm, through interference with endocrinological mechanisms. Estrogens are among the most potents for this purpose; but long-term administration of estrogens to men can cause breast enlargement, loss of libido, and increase in thromboembolic disease. Nevertheless, limited clinical investigations are still being conducted in the hope that these problems can be minimized by using very low doses of estrogens combined with testosterone. The preliminary results of these clinical studies confirm the observation first made by researchers in the mid-1970s that suppression of sperm production can be achieved in the short-term without evident side effects (Briggs, 1974 and Ewig, 1978). Any extensive development of this approach, however, is not likely available because of the toxicity of estrogens in long-term administration.

Although progestins are less potent than estrogen in inhibiting spermatogenesis, they have been much more widely tested in men because there is no correlation between their use and the incidence of cardiovascular disease in men. Sperm production can indeed be suppressed using progestins supplemented with androgens. Normal plasma testosterone levels are maintained, and sperm production can be restored when treatment stops. Current research continuing the

efforts begun a decade ago (Coutinho and Melo, 1973; Frick, 1973; Frick and Bartsch, 1974) still seeks to identify effective hormonal combinations without unacceptable side effects in dosages to be administered as either monthly injections or daily pills. Success is still elusive.

Sperm production can be inhibited by testosterone alone if a sufficiently high dosage is applied. Daily intramuscular injections are necessary for such action, but this may lead to long-term health hazards, particularly the vascular problems. In an attempt to reduce the toxicity, active steroids, which are less androgenic than testosterone, have been tested by oral administration (Cunningham *et al.*, 1978; Mauss *et al.*, 1974; Reddy and Rao, 1972; Steinberger and Smith, 1977). However, these anabolic agents were not so effective, even at a very high dosage (Leonard and Paulsen, 1978).

Many attempts have been performed over the years to develop reversible male contraceptives. Gossypol, a natural substance extracted from the cotton plants, appears to cause the suppression of sperm production or sperm motility. Its ability to reduce spermatogenesis in men is undeniable and has been demonstrated in both large scale studies in China (Wu, 1972; Wang and Lei, 1972; Dai *et al.*, 1972; Wang *et al.*, 1972; Shandong Coord. Group Antifertil. Plants, 1972; Zhang and Shi, 1972; Jiangsu Coord. Group

Male Antifertil. Agent, 1972 and Qian *et al.*, 1972) and a small scale confirmatory clinical trail in Brazil (Coutinho *et al.*, 1984; Coutinho and Melo, 1988). These investigations have suggested that gossypol can exert an effect on the testis's gamete-producing function, leaving its hormone-producing function undisturbed. Thus, it is possible to maintain normal testosterone levels and the libido state, but at the same time, inhibit sperm production and its motility. Because of this unique and important action, gossypol is worth to be fully investigated.

I. THE HISTORY OF GOSSYPOL

Gossypol was first discovered by J.J. Longmore in 1886 (Longmore, 1886) and was purified in crystalline form by the Russian chemist, L. Marchlewski, in 1889 (Marchlewki, 1889). People have often wanted to make use of all parts of the cotton plant. The seeds contain protein and oil, and attempts have been made to adapt the cotton meal as an animal feed or as infant food. However, the plant was found to be toxic and such attempts were not successful.

In the late 1960's, people in many rural areas of China, including the Hubei and Hebei provinces, had had complaints of fatigue and of burning of the faces, extremities, and other exposed parts of the bodies. The

farms in these areas grew cotton. The afflicted people who were unable to work in the fields, but hid in the shade or lay on rocks to get cool. When many local doctors were puzzled about the cause, the disease was spreading like epidemic. The peasants called this disease: "the burning fever" (Wu, 1972).

Burning fever was especially prevalent in Xingtai, a county in Hebei province. A local doctor discovered that these affected peasants consumed raw, homemade, cotton seed oil. Commercially manufactured cotton seed oil had been used in cooking for many years, but only in the 1960's, did the peasants begin to make oil from uncooked seeds, using their own compressing machines. Raw cotton seeds contain gossypol which can be destroyed by heat. Unlike the commercial process, preparation of the homemade oil has avoided the heating procedure. Consequently, gossypol remains intact in the homemade oil. This substance was discovered to be the cause of the burning fever (Wu, 1972).

As soon as the crude cotton seed oil was identified as the source of burning fever, Xingtai doctors advised their patients to stop using their own raw oil. After the doctors' advice was carried out in those patients, the burning and fatigue symptoms stopped. Several years later, however, many couples were found to be experiencing infertility problems. A large number of women had

amenorrhea. These cases of infertility were regarded as a sequel of the burning fever. When those women remained on gossypol-free diets, many eventually recovered from amenorrhea. For the men, despite the elimination of gossypol from their diets , only a few recovered from their infertility. Further examination of these men revealed azoospermia or oligospermia. In addition, some men exhibited a decrease in testicular size. Medical and scientific research workers from universities and hospitals were sent to the area to investigate the problem. They confirmed the findings of the local doctors. Infertility was prevalent, and women seemed to recover at a much higher rate than men. Men who did recover were found to have consumed a lesser amount of the cotton seed oil and only for a shorter periods of time than others. This information had led the investigators to study whether controlled doses of purified gossypol could be used effectively as a male fertility-control agent. Observational studies in the countryside had shown that burning fever, fatigue, and infertility were the most serious effects of gossypol ingestion. Mortality was not observed as a result of burning fever. As the rate of recovery from male infertility was dependent on the amount of cotton oil a man had consumed, scientists conjectured that infertility would most likely be reversible if the gossypol could be administered under a controlled dosage.

Ever since the Chinese National Coordinating Groups on Male Antifertility Agents announced in 1978 that gossypol could serve as a new potential contraceptive for males (National Coordinating Groups on Male Antifertility Agents, 1978), many works have been focused on the toxicology, pharmacokinetics, biochemistry and clinical trials of the compound. Over two hundred reports on gossypol have appeared since 1979 (Lei, 1983; Prasad *et al.*, 1982; Qian, 1981; Tso, 1985; Tso *et al.*, 1985; Shi *et al.*, 1987; Leung and Tso, 1988) In 1982, the Special Programme of Research in Human Reproduction of the World Health Organization (WHO) initiated an international programme for the synthesis and biological evaluation of gossypol analogues.

II. THE CHEMISTRY OF GOSSYPOL

Gossypol, $C_{30}H_{30}O_8$, has a molecular weight of 518.54 is termed as 1,1',6,6',7,7'-hexahydroxy-3,3'-dimethyl-5,5'-bis(1-methylethyl)[2,2'-binaphthalene]-8,8'-dicarboxaldehyde. The crystal of gossypol was first synthesized by Edwards (1958). It is polymorphic and its melting points varies with the solvents from which they are crystallized:

Solvent	Melting point
Diethyl ether	184°c
Chloroform	199°c
Ligroin	214°c

These polymorphic molecules have different optical properties and crystalline forms but show no differences in their chemical and spectral behaviour. It indicates that they may have similar chemical structures. Base upon the multiplicity of the reactions of gossypol, three tautomeric forms of gossypol were proposed (Adams and Geissman, 1960). They were the aldehyde, the ketonoid, and the hemiacetal forms (Figure 1-1). In ordinary inert solvents, gossypol exists mainly in aldehyde form, while in polar solvents, such as DMSO, the hemiacetal form occurs in dynamic equilibrium with the aldehyde form (Baram *et al.*, 1976).

Gossypol is insoluble in water, slightly soluble in petroleum ether, and soluble in common organic solvents such as methanol, ethanol, diethyl ether, chloroform and dimethylformamide. It is also freely soluble in dilute aqueous solutions of ammonia and sodium carbonate, but there may be slow decomposition on standing.

Recently, the properties of gossypol samples recrystallized from different solvents are investigated and compared (Jiang, 1986). Gossypol can be crystallized as lumps from petroleum ether (60-90°C). It appears as prism form when crystallized from chloroform. However, when crystallized from ether-ethanol-water (1:2:2), it appears as plates. The UV, IR and ^{1}H-NMR spectra of the different samples are tested and found to be dependent on their

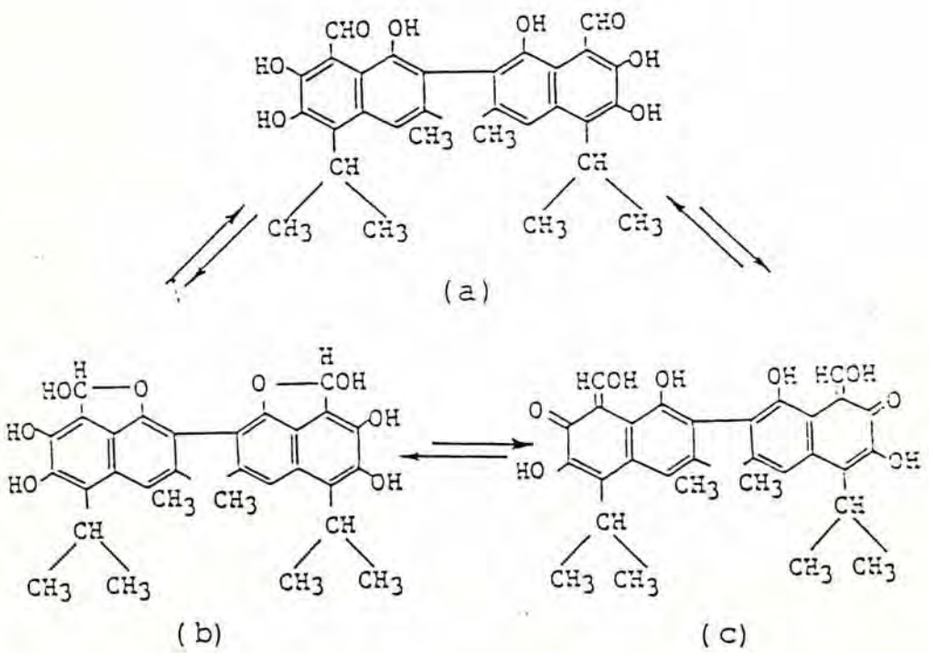

Fig. 1-1 Tautomeric forms of gossypol: a, hydroxy aldehyde; b, hemiacetal; c, ketonoid.

crystalline forms (lumps, prisms or plates) and molar concentrations. The polarographic behaviour of gossypol in various buffers has been studied by Jiang *et al.* (1984). They found that below pH 5, a well-defined reduction wave was obtained. When the solution was above pH 5, the reduction wave deteriorated and could not be measured accurately. A recent study on the chemistry and applications of gossypol by a Chinese group elucidated some of the problems related to the steric structures and tautomerisms of gossypol and its derivatives, as well as the separations of their isomers (Wu *et al.*, 1985).

A. Enantiomers of Gossypol

As a result of restricted rotation about the bond linking the two naphthyl residues, gossypol is a typical chiral molecule (King and deSilva, 1968). Gossypol isolated from cotton plant exists as a racemate, i.e., an equal mixture of the (+)- and (-)-enantiomers (Dechary and Pradel, 1971). Subsequently, the optically active (+)- form was firstly isolated from Thespesia populnea (Dechary and Pradel, 1971; Datta *et al.*, 1972) and again from Montezuma speciosissima (Marcelle *et al.*, 1985). Later, it can also be isolated from the resolution of racemic gossypol by chemical methods from the Gossypium sources (Zheng *et al.*, 1985). The spectral characteristics and the melting points of the (+)-isomer and the racemate are identical, but the

former is more soluble in ordinary organic solvents than the latter.

Since (+)-gossypol has been found to be inactive as an antifertility agent, there is a strong implication that (-)-gossypol is the only form that possesses the antifertility activity in the male (Lindberg *et al.*, 1987). Since then, a search for plants producing (-)-gossypol had been carried out. Recently, Chinese group reported to have isolated (-)-gossypol with optical purity of 12-25% from the seed of Gossypium barbadense (Malvaceae) (Zhou *et al.*, 1987). The other alternative is to separate the racemic gossypol into (+)- and (-)-enantiomers. Nowadays, separation of the racemate into (+)- and (-)-enantiomers had been succeeded by various groups (Si *et al.*, 1983; Zheng *et al.*, 1985; Matlin *et al.*, 1988). This has provided a powerful tool for the study of the biological actions of the two enantiomers. It was found that only the (-)-gossypol exhibited both contraception and toxicity *in vivo*. However, no difference had been found between the effects of the two enantiomers *in vitro* (Yao *et al.*, 1987).

B. Analysis of Gossypol

Gossypol is known to be unstable and often appears in the form of gossypol acetate, though, there are two other forms of gossypol, i.e. gossypol and gossypol formic acid.

They have been used in laboratory investigations and clinical trials. Gossypol is unstable in aqueous solution: either oxidized by air or transformed into other isomers depending on the pH (Adams and Geissman, 1960; Lee *et al.*, 1982). However, gossypol was found to be relatively stable under acidic pH (Lee *et al.*, 1982). But this has been challenged by the work of Leung and Tso (1988). They observed that gossypol pre-incubated at basic pH was eluted as a single peak by the reverse-phase HPLC system. As the pH decreased, more transformation peaks appeared. While the nature of the various separable moieties is unclear, the gossypol antifertility effect depends mainly on the amount of pure gossypol and not its impurities (Waller *et al.*, 1981). Therefore, standardised methods for the quantitative and qualitative analysis of gossypol are necessary.

Many methods have been suggested for the determination of gossypol in a variety of samples (Pons, 1977). They include spectrophotometry (Crouch and Bryant, 1982 and Admasu and Chandravanshi, 1984), fluorometry (Aver'yanov *et al.*, 1978), NMR (Waiss *et al.*, 1978), GLC (Raju and cater, 1967), polarography, TLC and paper chromatography (Markman and Rzhekhin, 1968). Although there are many methods for the determination of gossypol, the suitable mean depends on the conditions of various laboratories.

III. METABOLISM AND DISTRIBUTION OF GOSSYPOL IN BODY

Gossypol is absorbed through the intestine as well as the epithelial lining of the stomach (Abou-Donia *et al.*, 1970). Fecal excretion is the major route by which gossypol, administered either orally or parenterally, is removed from the animal body. Most of the absorbed gossypol is excreted via bile, suggesting biliary circulation of gossypol between the liver and the intestine (Lyman *et al.*, 1969; Abou-Donia *et al.*, 1970; Abou-Donia and Lyman, 1970; Albrecht *et al.*, 1972; Wang *et al.*, 1973; Abou-Donia and Dieckert, 1974; Abou-Donia and Dieckert, 1975; Xue *et al.*, 1975a; Xue *et al.*, 1975b; Tang *et al.*, 1980). High concentrations of gossypol in the bile are in harmony with the tentative conclusion that compounds of high molecular weight containing polar anionic groups and two or more aromatic rings tend to be excreted into the bile.

In rats, after a single oral dose of (^{14}C)-gossypol was administered for a 13-day experimental period, 77.4% of the ingested radioactivity was recovered from the feces, 12.1% from the expired CO_2, and 3.1% from the urine (Abou-Donia *et al.*, 1970). The accumulation of radioactivity in the body tissues was relatively low; only 12.5% of the administered dose was found in various tissues one day after the administration. Among the various tissues, gastrointestinal tract contained the highest radioactivity. The other various tissues listed in the decreasing order of radioactivity as follows: liver, heart, kidney, spleen,

lung, blood, muscle, adipose tissue, testis, and brain. After 24 hours, the radioactivities of all these tissues gradually decreased, and on the 13th day, the total tissue radioactivity was only 0.28% of the administered dose. The peak of radioactivity, in almost all the tissues examined, was at the 24th hour after administration. Reabsorption of gossypol from the renal tubule through the mechanism of nonionic absorption might account for the low urinary excretion of radioactivity. But a recent study had confirmed that gossypol was transported by the renal tubule through the classic organic anion system (Goldinger *et al.*, 1985). The half-life of gossypol in the rat body was approximately 60 hours. There were discrepancies in the metabolic pathways in various animal models (Tang *et al.*, 1980; Sang *et al.*, 1980). It is believed that this difference may be responsible for the differential responses to the antifertility and toxic effect of gossypol (Tang *et al.*, 1980; Shang *et al.*, 1980).

IV. THE EFFECT OF GOSSYPOL

A. Effect of Gossypol on Different Animal Species

The antifertility effect of gossypol had been studied in a number of laboratory animals. It was found that different animal species showed marked sensitivity variations towards gossypol. Among the laboratory animals

tested, hamsters seemed to be the most sensitive, followed by rats, monkeys, and dogs, while rabbits and mice appeared to be insensitive (Wang *et al.*, 1972; Natl. Coord. Group Male Antiferil. Agents, 1978; Shang *et al.*, 1980; Chang *et al.*, 1980; Hahn *et al.*, 1981; Saksena *et al.*, 1981). The effective dose for hamsters was 5-10 mg/kg/day, given for 6-12 weeks. Recovery of fertility occurred 4-14 weeks after withdrawal of the drug (Chang *et al.*, 1980; Hahn *et al.*, 1981; Waller *et al.*, 1981; Saksena and Salmonsen, 1982). The effective dose for rats ranged from 10 to 30 mg/kg/day, given for 3-10 weeks. Recovery of fertility occurred 3-12 weeks after withdrawal of the drug (Qian and Wang, 1984). Long term treatment might cause complete atrophy of the seminiferous epithelium in some of the animals, and permanent sterility is the likely consequence. In dogs, gossypol could inhibit spermatogenesis only at a high toxic dose (Shang *et al.*, 1980).

Monkeys are moderately sensitive to the anti-spermatogenic action of gossypol. When rhesus monkeys were given a dose of 4 mg/kg/day for two years, spermatogenesis was completely inhibited in two of the three animals; in the third, a few normal spermatids and spermatozoa could still be found in some of the seminiferous tubules (Shang *et al.*, 1980). In cynomolgus monkeys, gossypol at a dose of 10 mg/kg/day given for as long as 6 months only decreased the sperm count and motility in the ejaculate (Shandilya *et*

al., 1982).

In rabbits and mice, gossypol did not induce infertility. When rabbits were given gossypol at a dose of 10 mg/kg/day for 14 weeks, no significant difference compared with control was observed in the sperm concentration and motility in the ejaculate (Chang *et al.*, 1980). Even though the duration of treatment was prolonged to 250 days, both the sperm counts and fertility were not significantly affected and the rabbits eventually died (Saksena *et al.*, 1981). In mice, an oral dose of 15-30 mg/kg/day did not significantly affect the motility of spermatozoa (Shi and Zhang, 1980); the same results in rabbit was found (Hahn *et al.*, 1981).

B. Effect of Gossypol on Male Reproductive Organs

1. Testis and epididymis

In rats fed with a contraceptive dosage of gossypol for more than one month, a local degeneration of the seminiferous tubules as well as a decrease in sperm count were observed in parallel with a change in the basement membrane of the germinal epithelium. The epididymis of the gossypol treated rats were usually lighter in weight than those obtained from the control animals.

The spermatids and spermatocytes of mid- and late-

stages were almost absent from the seminiferous tubules of gossypol treated rats. At its severity, the damaged seminiferous tubules became atrophic and depopulated to a marked degree, with only a single layer of cells consisting of Sertoli cells and spermatogonia left in the tubules. Moreover, the storage of dead spermatozoa in the caudal epididymis led to the presence of phagocyte.

On the contrary, the Leydig interstitial cells and the epididymal epithelia in gossypol treated rats were not affected. And the weight of the accessory glands as well as its morphology were normal (Dai *et al.*, 1978; National Coordinating Group on Male Antifertility Agents, 1978 and Xue *et al.*, 1981). However, Wong and Tam (1983, 1988a, 1988b) have reported recently that gossypol acetic acid has an inhibitory effect on the secretory activity of the seminal vesicle and the prostate. While the morphology of the Leydig cells was not affected by gossypol administration, serum testosterone level was reduced to 20% of that of the control animals. At the same time, diminution of testosterone synthesizing capacity was retained but at a lower level than the control (Hoshiai *et al.*, 1981 and 1982). Studies employing tracer techniques, vascular perfusion and intratesticular perfusion of horseradish peroxidase showed that the spermatogenic cells were severely damaged by gossypol treatment while the permeability of the blood-testis-barrier was not affected

(Yang *et al.*, 1982). It is obvious that gossypol does not interfere the blood-testis-barrier.

Although it has been reported that gossypol administration did not impair protein synthesis in the sertoli cells (Zhou, 1982), ultrastructural changes related to phagocytic activities were reported (Wang *et al.*, 1982). These changes included an increase in the number of lysosomes, lipid droplets, ring or cup shaped mitochondria as well as the multiform changes of mitochondria and lysosomes. After six weeks of treatment, the sertoli cells began to show degenerative changes such as distension and vesiculation of endoplasmic reticulum, accumulation of lipid droplets, cellular debris and lysosomes in varying sizes and phases, and the occurrence of atrophic changes in mitochondria were noted.

2. Spermatozoa and spermatogenic cells

The effect of gossypol on spermatozoa both *in vivo* and *in vitro* constitutes a substantial portion of literature about gossypol effects. The spermatogenic cells are the target cells of gossypol action and studies of gossypol effect would help to resolve the mechanism of the contraceptive effect induced by gossypol.

The changes induced by gossypol can be classified into structural, motility and biochemical aspects. In rat, the

spermatids and spermatocytes of the mid- and late-stage were most sensitive and vulnerable to gossypol inhibition. Ultrastructural observations of spermatozoa from gossypol treated patients revealed that the mitochondrial sheath and the acrosomal cap were the first to show detectable damage and suffered the most severe damage (Xue, 1980). The axial filaments and nuclei also demonstrated different degree of abnormalities. The severely damaged spermatozoa finally disintegrated, followed by appearance of cell debris and exfoliated immature spermatogenic cells (Hang *et al.*, 1980). Spermatozoa from the cauda epididymis exhibit distinctive changes including wrinkled and disorganized cell membrane in the head and tail region, cell membrane missing from segments of the tail midpiece and principal piece regions, malformed heads, decapitated spermatozoa, retention of cytoplasmic droplet at variable loci along tail midpieces, and looped tails. Hoffer (1982) noticed a significant damage of virtually all sperm flagella throughout the epididymal duct of gossypol treated rats. Again, the initial and prominent defect was the degeneration of the midpiece mitochondria while the ultrastructure of the epididymal and vasal epithelium was not affected. It was suggested that gossypol exerted its contraceptive effect during spermatogenesis and spermiogenesis, including the post-testicular development and maturation of spermatozoa in the epididymis (Bozek *et al.*, 1981).

It is well known that gossypol inhibits both *in vivo* and *in vitro* spermatozoal motility. Hadley *et al.* (1982) showed that the injection of gossypol into the fat pad adjacent to the caput epididymis inhibited sperm motility *in vivo*. In the *in vitro* studies, 90% inhibition of the human sperm motility was achieved by 1.72 nanomolar gossypol (Ridley and Blasco, 1981; Kalla and Vasudev, 1980). The loss in motility probably was not due mainly to a lack of energy supply since it was noticed that inhibition of motility had a lower threshold than that of the sperm flagella and mitochondria ATPase (Tso and Lee, 1982a). It was also claimed that the inhibition of motility probably was not due to a leakage of potassium from the spermatozoa since the latter process had a much higher threshold than that of motility (Tso and Lee, 1982b).

It should also be bore in mind that gossypol uncoupled the respiratory chain and oxidative phosphorylation of boar spermatozoa resulted a stimulation of respiration at low concentrations and an inhibition at high concentration *in vitro* (Tso and Lee, 1981 and 1982c).

C. Effect of Gossypol on Reproductive Hormones

Gossypol does not seem to affect hormone directly level as a primary target of contraception. Both the plasma level of luteinizing hormone (LH) and testosterone were not

affected by low dosages of gossypol administration (Wang *et al.*, 1979; Natl. Coord. Group Male Antifetil. Agent, 1978; Kalla *et al.*, 1982). Also the response of the pituitary to luteinizing hormone releasing hormone (LHRH) and follicle-stimulating hormone (FSH) binding by testis were normal when compared with control. On the other hand, Liang *et al.* (1981) reported a decrease in plasma testosterone level in gossypol treated rats (20 mg/kg/day for two months), and suggested that gossypol might exert a direct effect on the Leydig cells.

Four types of gonadotrophs in the rat adenohypophysis can be identified under electron microscope. The control groups mainly consisted of types I and II gonadotrophic cells with the latter constituting about 65% of the total. Following gossypol adminstration, the number of type II cells decreased and the number of type III cells increased to become the principal cell type in the gossypol-treated animals (58% of total gonadotrophs). Type III cells were characterized by the appearance of dilated vesicle derived from rough endoplasmic reticulum which occupied almost all the cytoplasm. Morphologically, these cells were similar to castration cells which appear in the rat adenohypophysis after gonadoectomy. No ultrastructural changes were observed in the interstitial cells of gossypol-treated rat (Ye *et al.*, 1982). In hamster, the serum concentration of testosterone, prolactin and LH were not affected by

gossypol administration. A dosage of 15 mg/kg/day for 8 weeks led to infertility while the serum level of the reproductive hormones were not altered (Saksena and Salmonsen, 1982). In the monkey (Macaca fascicularis), administration of gossypol at a dosage of 10 mg/kg/day for 6 months resulted in a decrease of sperm concentration and motility but the plasma levels of testosterone was not affected (Shandilya *et al.*, 1982). Similarly, pigs fed with gossypol also showed normal serum FSH level. Rabbits, which are insensitive to gossypol, were found to show slight fluctuation in serum FSH level when fed with this compound (Wang *et al.*, 1979; Natl. Coord. Group Male Antifertili. Agent, 1978). However, in human subjects, the serum testosterone level remained the same after gossypol administration. The response of the testis towards LHRH and HCG was not altered by gossypol.

D. Effect of Gossypol on Enzymes

Gossypol is highly reactive with proteins (Lyman *et al.*, 1959). The carbonyl group of gossypol readily react with the free ϵ-amino groups of lysine residues to form Schiff's base and cannot be removed by dialysis (Tanksley *et al.*, 1970). Due to its high reactivity, gossypol was shown to inhibit large number of enzyme activities *in vitro*.

1. Enzymes in capacitation

Sperm capacitation in the guinea pig had been reported to be inhibited by gossypol (Shi and Friend, 1983). But the inhibition could be reversible if spermatozoa were washed free of gossypol by two centrifugation steps at 500 g for 10 minutes after gossypol treatment. Once the spermatozoa were capacitated in gossypol-free medium the gossypol did not block the acrosome reaction.

The gossypol effect on sperm capacitation of human spermatozoa were also investigated *in vitro* by testing the penetration ability of the gossypol treated sperm to zona-free hamster egg. It was found that gossypol treatment caused a dose dependent decrease in the ability of sperm to become capacitation and of penetrating the hamster oocytes (Kennedy *et al.*, 1983; Aitkin *et al.*, 1983). It was also suggested that the total amount of acrosin activity, a proteinase required during fertilization, was significantly diminished in the gossypol treated spermatozoa in a dose-dependent manner, which was correlated closely with the decrease in oocyte penetration. Further detailed study was performed on the interactions of gossypol with acrosin, by using a purified and well characterized proacrosin-acrosin system from the boar. Under conditions similar to those used in the penetration experiment, gossypol prevented the conversion of proacrosin, the zymogen form of acrosin which predominated in freshly ejaculated spermatozoa, into active

acrosin. Gossypol was also suggested to block spermatozoal penetration of egg *in vivo* by inhibiting hyaluronidase as well as proteolytic enzymes (Shi, 1986).

2. The metabolic enzymes

Gossypol exhibited an inhibitory effect on sperm fructolysis and glycolysis (Poso *et al.*, 1980; Stephens and Critchlow, 1982; Wichmann *et al.*, 1983), and caused a depletion of intracellular ATP concentration (Ke and Tso, 1982). It might be resulted from the effect of gossypol on various enzymes related to spermatozoal enzyme metabolism.

a. On glycolysis

Among the glycolytic enzymes, hexokinase, which was a regulatory enzyme working in an irreversible manner under physiological conditions, was studied because of its key role in glycolysis. In some *in vitro* studies, hexokinase was found to be insensitive to gossypol at concentration up to 5.6×10^{-5}M. A significant inhibitory effect was observed only at or above a concentration of 10^{-4}M. 50% inhibition was found at 1.3×10^{-4}M. When the gossypol concentration was 1.8×10^{-4}M, only 10% of the hexokinase activity remained.

In the final step of glycolysis, pyruvate is reduced to lactate with the regeneration of NAD^+. This process is catalyzed by lactate dehydrogenase (LDH). Under some conditions, LDH can also play an important role in the

reverse reaction which channels lactate to the tricarboxylic acid cycle. In testis, and in particular spermatozoa, a special LDH isoenzyme, namely LDH-X was found (Blanco and Zinkham, 1963). Lee and Malling (1981) first showed that gossypol could inhibit selectively LDH-X in mice and human. Later, Tso and Lee (1982d) examined the activities of LDH-X of boar spermatozoa in the presence of gossypol and also found that LDH-X was more sensitive to gossypol than the other LDH isozymes. Similar results were also reported in rat spermatozoal LDH-X and purified LDH-X from bovine testis (Giridharan *et al.*, 1982; Olgiati *et al.*, 1983). Gossypol and some of its analogues have been shown to be potent inhibitors of LDH-X. It was suggested that LDH-X might serve as a model for understanding the gossypol binding sites and its contraceptive action (Whaley *et al.*, 1986). However, it was observed that LDH-X was equally susceptible to both (+)- and (-)-gossypol inhibitory action. Beside, LDH-X prepared from rabbit sperm was also inhibited by gossypol. These have led to a speculation that the inhibitory effect of gossypol on sperm LDH-X activity may not be a major mode of action for its antifertility action (Eliasson and Virji, 1983; Shi *et al.*, 1987). In contrast, Tso suggested that the inhibition of LDH-X by gossypol might still be one of the many factors caused infertility when they acted together (Shi *et al.*, 1987).

b. On TCA cycle

The prevention of CO_2 formation from (1-^{14}C) and (2-^{14}C) pyruvate indicated gossypol might have a direct effect on the sperm TCA cycle (Wichmann *et al.*, 1983). Several metabolic enzymes participating in the TCA cycle in sperms had also been reported to be inhibited by gossypol (Tso and lee, 1982e). Table 1-1 showed the effect of gossypol on the activities of enzymes of the TCA cycle. Among the enzymes, aconitase and fumarase were most sensitive to gossypol (Shi *et al.*, 1987).

c. On oxidative phosphorylation

Gossypol has been recognized as an uncoupler of oxidative phosphorylation (Abou-Donia and Dieckert, 1974; Nakamura *et al.*, 1988). At low concentration, it stimulates aerobic respiration but at high concentration, it inhibits the respiration (Tso and Lee, 1981) and reduces ATP production (Kalla and Vasudev, 1981; Tso and Lee, 1982e). It has been suggested that gossypol may exert its toxic effect in animals by uncoupling oxidative phosphorylation.

3. Adenyl cyclase

Adenyl cyclase is a key regulatory enzyme in spermatogenesis, which is involved in production of cyclic AMP and is required for the maturation of germ cells in testis and epididymis. It had been demonstrated that adenyl cyclase was inhibited by gossypol in a dose-dependent

Table 1-1 Effect of gossypol on TCA cycle enzyme activites[a]

Enzyme	Gossypol effective concentration (M)		
	Maximum stimulation[c]	Inhibition[b]	
		IC_{20}	IC_{50}
Citrate synthase	----	2.4×10^{-4}	3.2×10^{-4}
Aconitase	10^{-5} (110%)	2.0×10^{-5}	3.7×10^{-5}
NAD-isocitrate dehydrogenase	----	2.4×10^{-5}	1×10^{-4}
α-Ketoglutarate dehydrogenase	10^{-5} (127%)	1.8×10^{-4}	----
Succinyl-CoA synthetase	----	1.8×10^{-5}	5.6×10^{-5}
Succinate dehydrogenase	10^{-5} (123%)	2.4×10^{-4}	----
Fumarase	----	3.7×10^{-5}	4.9×10^{-5}
NAD-malate dehydrogenase	----	7.5×10^{-5}	1.8×10^{-4}

a. adopted from Hi *et al.*, 1987. b. Various % inhibition concentrations are given. $IC_{20,50}$ means 20% activities have been inhibited. c. Numerical values in brackets represent the % activity of the control.

manner (Olgiati *et al.*, 1984; Vishwanath and White, 1986). Since sperm adenyl cyclase required Mn^{2+} as a cofactor, the inhibition was shown to be mediated through chelating Mn^{2+} by gossypol (White *et al.*, 1988). The inhibition of adenyl cyclase will decrease the intracellular cyclic AMP level, leading to lower protein kinase activity and results in sperm dysfunction.

4. Na^+/K^+-ATPase

Renal Na^+/K^+-ATPase activity in guinea pigs was demonstrated to be inhibited by gossypol when the animal was fed with a relatively low potassium diet (Bi *et al.*, 1980). In mild antifertility dosage, renal Na^+/K^+-ATPase activity was found to be unaffected in rats, guinea pigs, rabbits, and monkeys when fed with normal diets (Qian and Wang, 1984). However, when large dosage were given to guinea pigs, both renal and skeletal muscle Na^+/K^+-ATPase activities were inhibited (Su *et al.*, 1982). The Na^+/K^+-ATPase activities in the brain synapses of rat and the renal cortex of guinea pig and sea urchin spermatozoa were also inhibited by gossypol in dose-dependent manner (Adeyemo *et al.*, 1982; Feng and Xu, 1982; Ye *et al.*, 1983). This inhibition of Na^+/K^+-ATPase was suggested to be due to hypokalemia caused by gossypol (Qian *et al.*, 1975; Bi *et al.*, 1980; Qian, 1981).

E. Other Effects of Gossypol

Gossypol is a compound that exhibits a broad spectrum of biochemical actions. At the membrane level, gossypol affects the electrochemical properties of lipid membrane and the orderliness of membrane lipid matrix (Reyes *et al.*, 1984 and 1986). Other biological effects of gossypol include anti-viral activity (Wichmann *et al.*, 1982) and anti-parasitic activity (Eid *et al.*, 1988). Recently, human immunodeficiency virus (HIV) has been reported to be inactivated by gossypol in *in vitro* studied (Polsky *et al.*, 1989).

In view of the wide range of diversified gossypol interactions with the biological system, its anti-tumour actions have also been examined. Gossypol was able to lengthen the survival of 10-12-week-old C57 BLxDBA/2F_1 mice bearing mouse mammary adenocarcinoma 755 (Rao *et al.*, 1985). It was also effective in suppressing the growth of TR-ST cells originated from a rat testicular tumour (Tanphaichitr *et al.*, 1984). NMRI mice implanted with Ehrlich ascites tumour cells were able to survive longer than control after gossypol administration (Tso, 1984). Recently, it had also been confirmed that gossypol concentration over 0.5 μM reduced the growth rate of the human SW-13 adrenocortical carcinoma cells *in vitro* (Wu *et al.*, 1989).

V. TOXICITY OF GOSSYPOL

A. General Toxic Effect

Tolerance to gossypol varies greatly and is dependent on species. LD_{50}'s of gossypol in aqueous solution, given orally at a single dose, for some animals are listed in the Table 1-2; if the drug was dissolved in oil, the LD_{50}'s would be 10% less than that in aqueous solution. For multiple dose response in mice, rabbits, rats, guinea pig, dogs, pigs, hamsters and monkeys, rats and hamsters seem to the most tolerant species, while dogs and rabbits are the least. Daily oral doses as low as 1.5 mg/kg given for 28 days could cause death in swine (Tollet *et al.*, 1957). Exposure of dogs to 1.0-3.0 mg/kg daily dose of gossypol could lead to severe toxic effect and eventual death (Eagle, 1950). Exposure of rabbits to a 16 mg/kg daily dose for a period of 140 days had led to bradycardia and ECG changes in some of the animals. Six out of ten animals died during the treatment period (Shandong Coord. Group Male Antifertil. Plants, 1973). The data obtained for liver enzymes and other blood components in animals administered with gossypol, including rats, rabbits, dogs and monkeys, seemed inconsistent and non-conclusive (Qian and Wang, 1984). It appears that different species will have variable response to gossypol toxicity (Waller *et al.*, 1986). At present, no animal has been found to develop hypokalemia following gossypol administration. Thus, one has to be very careful to extrapolate data obtained from animal models to

Table 1-2 Single-dose oral LD_{50} of gossypol (mg/kg) in water for several species

Species	LD_{50}
Rat	2400 - 3340
Mouse	500 - 950
Rabbit	350 - 600
Guinea pig	200 - 300
Pig	550

adopted from Qian and Wang, 1984

the study in human.

B. The Mutagenicity

Gossypol administered in 5- and 30-fold more than the usual clinical dosage in Wistar rats and long-hair rabbits respectively did not cause any embryotoxic or teratogenic effect (Tan *et al.*, 1982). Recently, however, gossypol had been observed to cause DNA strand breakage in human lymphocytes (Chen *et al.*, 1986). An Indian group had found that incubation of intact rat liver nuclei with gossypol promoted DNA degradation *in vitro*. Moreover, nuclei isolated from animals administered with gossypol also showed higher susceptibility to DNA fragmentation (Srivastava *et al.*, 1987).

Study using Ames test revealed that gossypol was not a mutagen (Colman *et al.*, 1979). Cells treated with gossypol *in vitro* showed no effect on chromosome breakage and chromatid exchanges (Bhagirath and Kunclu, 1985; Kainz *et al.*, 1985). However, gossypol became cytotoxic on continuous exposure. The direct effects included reduced mitotic index and the decrease in rate of synthesis of DNA, RNA and nuclear basic proteins (Fei and Teng, 1988; Teng and Fei, 1988; Zhang *et al.*, 1989). Another report indicated that it was a specific inhibitor on DNA synthesis. Since it irreversibly blocked cells in the S

phase, it could be applied as an anti-neoplastic drug (Wang *et al.*, 1984). Animal studies did indicate that gossypol inhibited Ehrlich ascites tumour cell proliferation in mice (Tso, 1984).

VI. CLINICAL TRIALS OF GOSSYPOL

The initial clinical trial of gossypol as a male antifertility agent was carried out by Qian *et al.* (1972). They found that gossypol given orally at a dose of 60-70 mg per day per person for 35-42 days caused a gradual increase in the percentage of non-motile spermatozoa in the ejaculate in all 25 volunteers. This was followed by oligospermia, necrospermia, and azoospermia in the subjects. Interestingly, sperm motility decreased markedly as early as from the second week onwards, suggesting that gossypol might have acted on epididymal or testicular spermatozoa. Recovery occurred around three months after the drug withdrawal. The side effects at this dosage were reversible and generally of mild degree. These included the loss of appetite, fatigue, dryness of mouth, diarrhoea, inconsiderable elevation of SGPT, and a tendency to sleepiness. Some individuals suffered slight oedema of the eyelid, seemingly decreased libido and potency, and a little decrease in serum potassium levels (Qian *et al.*, 1972).

After a series of toxicological studies, a second and a third phase trials were carried out in several parts of China. Until 1980, the total number of volunteers had amounted to 8806 (Liu *et al.*, 1981). Optimal (routine loading) and maintenance doses were determined to be 20 mg/day for 60-70 days and 40-50 mg/week respectively. With this dosage, antifertility efficacy was estimated to be 99.07%. The common side effects in men taking routine dose were similar to those reported by Qian *et al.* (1972). In addition, an infrequent but dangerous side effect, hypokalemic paralysis, was uncovered during the expanded trials.

Later, small scale clinical trials were performed in Brazilian and Austrian volunteers (Frick and Danner, 1985; Continho and Melo, 1988). Both studies has confirmed that gossypol inhibits spermatogenesis in men at a dose level for short-term treatment (up to 12 months) appears to be free of side effects.

The recovery of sperm count to normal level was observed in 74% of the subjects within 1 to 3 years after the treatment was stopped. The degrees of recovery, in general, were related to the total dose taken and the duration of the treatment (Natl. Coord. Group of Male Antifertil. Agent, 1985).

In order to identify the smallest effective gossypol dose in man for fertility control, subjects were administered with gossypol, 10 mg/day, orally for 3 months. The forward sperm motility and sperm density were markedly decreased with no observable side effects (Frick *et al.*, 1988).

VII. THE PURPOSE OF THIS STUDY

Though gossypol has been studied extensively and intensively on the toxicology, pharmacokinetics, biochemistry and clinical trials for over ten years, the mechanism underlying its actions is still unknown. This study explores two aspects of this unknown mechanism, one is the clarification of the role of LDH-X in gossypol antifertility action and the other is the effect of gossypol on the antioxidant defense system of the animal. Along with such studies, some preliminary investigations on the effect of diet on gossypol efficacy and gossypol on zinc metabolism are also included.

A. Gossypol and LDH-X

Lactate Dehydrogenase-X (LDH-X), a spermatozoal specific enzyme in the energy production of germinal cells, has been suggested to be the target enzyme of gossypol. But such a speculation has not been supported by accumulating

data (see early parts of this introduction). In this study, we want to examine the existence of LDH-X in other fowls which are used to be investigated so as to provide a ground for the study of the gossypol effect in these subjects.

Moreover, it was reported that LDH-X band could not be found in the starch gel electrophoresis of the testis of cock, drake, hug and cat. If this is a fact and that LDH-X is the only target enzyme of gossypol, then the spermatogenesis of cock and drake should be resistant to gossypol. We hence measured LDH-X on the sperm and sperm-generating cells of these two fowls using the method established in our laboratory along with the antifertility effect of gossypol on these two fowls. This preliminary study will provide fundamental information for future work on the role of LDH-X in gossypol antifertility action.

B. The Effect of Gossypol on Antioxidant Defense System

Ultrastructural examination of epididymal spermatozoa and testicular spermatids in gossypol-treated rats demonstrated marked damage to the tail region, especially pronounced in the mitochondrial sheath of the midpiece. A high incidence of segmental aplasia of the mitochondrial sheath was found in both epididymal and testicular sperm. And the special double-membrane structure of mitochondrial cell membrane is more susceptible to free radical damage.

A recent *in vitro* study examining human sperm and rat liver microsomes found that gossypol promoted the formation of reactive species of oxygen such as hydrogen peroxide and superoxide radicals. Since cell death owing to reactive species of oxygen is likely to be the combination of an overwhelming production of free radicals and a compromised antioxidant defense system, this leads to a belief that the effect of gossypol may be related to free radical injury. Therefore, in this study, we examine the effect of gossypol on the antioxidant defense systems of hamster. At the same time, we tested some free radical scavengers in the testes by injecting these chemicals intratesticularly to the hamsters. The latter study is to show indirectly the effect of gossypol on free radical physiology.

C. The Influence of Gossypol on Zinc Metabolism

Zinc is a trace element that plays a variety of physiological roles, especially for the maintenance of spermatogenesis. In the view of its chemical structure, gossypol can act as a chelating agent and it can bind with divalent ions easily. Therefore, it is very necessary to clarify the relationship between gossypol and zinc, which may play a role for the explanation of the mechanism of gossypol action. Although the relationship between gossypol and iron have been well known, its relation to zinc metabolism is basically a blank. In order to estimate the

above relationship, the change of zinc content in the testis, hair and retina in hamster and mice was observed. It is hoped that the above studies may provide some fundamental information for further studies of the mechanism of gossypol induced antifertility.

CHAPTER 2 EFFECT OF GOSSYPOL ON LDH-X OF COCK AND DRAKE

I. INTRODUCTION

A. General Description of Lactate Dehydrogenase

In the final step of anaerobic glycolysis, NADH generated by glycolysis cannot be reoxidized by O_2 but can be reoxidized to NAD^+ by pyruvate, converting the latter into lactate. The reduction of pyruvate is catalyzed by lactate dehydrogenase (LDH) [EC 1.1.1.27]. NADH can be reoxidized under anaerobic condition, thus making LDH an important factor in anaerobic condition. In addition, LDH also plays an important role in the oxidation of lactate to pyruvate which is then channeled to the tricarboxylic acid cycle.

LDH and its isozymes are found in nearly every tissue, but the distribution of LDH activity among the isozymes is specific for each tissue (Allen 1961). LDH is a tetrameric enzyme of two somatic subunit types, A (or H) and B (or M). This gives a total of 5 homotetrameric and heterotetrameric molecules. The A type is usualy isolated from cardiac muscle and is abundant in tissues with aerobic respiration, whereas the B type, from skeletal muscle, is abundant in tissues with anaerobic respiration. It is now well established that each of these subunits is a separate gene

product and that the differential expression of these genes is responsible for the distinctive LDH isozyme pattern of different tissues. Therefore, by this theory, isozymes 1 through 5 have the following subunit composition: AAAA, AAAB, AABB, AAAB, and BBBB (Markert, 1962; Cahn, 1962; and Blanco, 1963).

B. Discovery of Lactate Dehydrogenase-X

Besides the five common LDH isozymes, a special LDH isozyme, designated lactate dehydrogenase-X (LDH-X) [EC 1.1.1.27], was found exclusively in testes and spermatozoa and sperm-generating cells of mammalian system (Blanco and Goldberg, 1963). LDH-X was first reported an extract of human testes and human spermatozoa more than two decades ago since being shown to be an additional zone of enzyme activity on the electropherograms (Blanco and Goldberg, 1963). This isozyme was believed to be a tetrapolymer of a polypeptide which is different from but closely related to the A and B polypeptides in molecular structure based on the finding that a dissociation-association studies of the enzyme mixture from the testis homogenate gave rise to new bands of activity due probably to different polymerization (Zinkham *et al.*, 1963; Goldberg, 1965). The monomeric polypeptide, or the C chain, might be formed by *de novo* synthesis or by posttranslational modification of the A or B polypeptides. If a third gene for the synthesis of the C

chain exists, the cistron is presumably active only in the testes and, more specifically, only active during spermatogenesis (Goldberg and Hawtrey, 1967). The existence of more than one X bands in some animals (Blanco *et al.*, 1964; Zinkham *et al.*, 1963 and 1964; Geogiev, 1982) suggests either that these isozymes are composites of more than one new polypeptide, or that the C polypeptide has combined with A or B subunits. Although Zinkham *et al.* (1963) favoured the first possibility, results of recombination studies on homogenates from guinea pig testes showed that one of the unusual LDH band is a heteropolymer of A and C chains, and possibly an A_2C_2 combination (Battellino and Blanco, 1970). Existence of only one C chain was supported by the fact that only one LDH-X band was found in epididymal and spermatozoal extracts from the guinea pig (Ballellino and Blanco, 1970), whereas multiple LDH-X bands were found in the testes homogenate of the same species (Zinkham *et al.*, 1963). It was proposed that only the C chain was synthesized in mature spermatozoa so that polymerization with other subunit types was impossible (Battellino and Blanco, 1970).

C. Properties of LDH-X

Unlike the other five cytosolic LDH isozymes, approximately 40% of the cellular LDH-X is localized in the mitochondrial fraction of sperm, and the remainder is

cytosolic (Montamet and Blanco, 1976). It is a unique LDH isozyme that can use α-ketovalerate or α-hydroxyvalerate as the substrate while other LDH isozymes cannot. Because of this substrate specificity, LDH-X can be assayed with α-ketovalerate as substrate in the presence of other LDH isozymes (Koeb *et al.*, 1970; and Blanco *et al.*, 1976).

According to the biochemical studies, LDH-X has been shown to be quite different from LDH-1 and LDH-5 in many of its kinetic characteristics, including pH optimun, temperature sensitivity, equilibrium constant (Km value), molecular weight and the sequence of amino acid. The pH optimun of LDH-X is 8.75-9.00, while that of LDH-1 and LDH-5 are 10.75 and 9.75 respectively. The Km value of human testes LDH-X for lactate is 25 mM, a value greater than that of LDH-5 (18 mM) and LDH-1 (2.9 mM) (Clausen and Ovlisen, 1965). The molecular weight of mouse's LDH-X is 140,000. The subunit is composed of 331 amino acid. After treatment of the mice isozymes at 65°C for 30 minutes, LDH-5 activity was totally inactivated and LDH-1 activity was 50% inactivated, while that of LDH-X activity was not affected at all (Hawtrey and Goldberg, 1970).

D. Role of LDH-X in the Spermatozoa

LDH-X activity is closely associated with sperm motility, and it has been proposed that the physiological

role of this isozyme is to shuttle reducing equivalents from the cytosol to the mitochondrial respiratory chain similar to the malate shuttle (Milkowski and Lardy, 1977; Clavin and Tubbs, 1978; and Blanco, 1980). The 2-oxo acids which are specific substrates of LDH-X can act as acceptors of hydrogen equivalents from NADH produced in the cytpolasm during aerobic glycolysis or oxidation of exogenous lactate. The reaction is catalysed by the 'soluble' LDH-X. The 2-hydroxy acids thus formed, penetrate into the mitochondria, where they can transfer the reducing equivalents to NAD^+ of the mitochondrial pool in the reaction catalysed by mitochondrial LDH-X (Blanco *et al.*, 1976). Therefore, if there is any damage to the LDH-X activity, it might affect the oxidation of NADH and the sperm motility might in turn be decreased.

E. Gossypol and LDH-X

Gossypol has been reported to be a potent non-steroidal male contraceptive agent (National Coordinating Group for Male Contraception, 1978). At the ultrastructural level, gossypol has been shown to produce extensive damage to the sperm mitochondrial sheath (Hoffer, 1982), while on the biochemical level, gossypol inhibits several enzymes involved in energy metabolism including Mg^{2+}/Ca^{2+} ATPase and several dehydrogenases (Kalla and Nadvasudev, 1981; Tso *et al.*, 1982a and 1985). That means the antifertility effect

of gossypol might be related to an inhibition of energy production.

Lee and Malling (1981) first showed a selective inhibition of LDH-X by gossypol in mice and human. This inhibition was further demonstrated by Tso and Lee (1982) *in vitro* on boar spermatozoa. Other reports on gosspol inhibition of rat spermatozoal LDH-X and LDH-X purified from the bovine testis have been accumulating since these years (Giridharan, 1982; and Olgiati, 1983). Based on these *in vivo* and *in vitro* studies of various animal species and human beings, the inhibition of the testis and sperm specific isozymes LDH-X as a target enzyme of gossypol was proposed to be the antifertility mechanism of gossypol.

On the other hand, Zinkham, Blanco and Clowry (1964) had reported that the testes of duck, chicken, hog and cat showed no LDH-X. If it is true, then all of these animals should be insensitive to the antispermatogenic effect of gossypol. If it is not true, it would be interesting to investigate whether there is really no LDH-X in the testes of cock, drake, hog and cat. Based on this idea, the finding of LDH-X activities in cocks and drakes will provide an extra tool in studying gossypol mechanism. At the same time, we can use another point of view to prove whether LDH-X is the real target enzyme of gossypol or not.

II. MATERIALS AND METHODS

A. Reagents

All chemical reagents used in the experiments were of analytical grade. The following reagents were purchased from Sigma Chemical Co.(U. S. A.): beta-nicotinamide adenine dinucleotide (reduced form); alpha-ketovaleric acid (sodium salt); rotenone; triethanolamine; copper sulfate; potassium sodium tartrate; crystalline bovine serum albumin. Phenol reagent (Folin-Ciocalteus) was purchased from Merck Chemical Co. (West Germany). The following chemicals were purchased from Riedel-de Haen Chemical Co. (West Germany): sodium chloride; potassium dihydrogen phosphate; di-sodium hydrogen phosphate. Pure gossypol (content 100.6%, by spectro-photometry; melting point 172.4°C) was a gift from Yu (Shanghi Institute of Physiology, Academia Sinica).

B. Methods

1. The effect of gossypol on the spermatozoa of cock and drake

Twelve cocks (Gallus Domesticus) weighing ca 1.5 kg, were purchased from local market. They were divided into two equal groups. One acted as the experimental group, while the other served as the control group. All the

experimental cocks were administered with gossypol (capsule), 10 mg/kg daily, 6 times a week. The control cocks were administered with empty capsule alone. During the experiment, the semen was artificially collected and observed under the microscope as described in appendix 1 once a week for 11 weeks. At the end of experiment, all cocks were sacrificed by vasotomy in jugular vessel.

Fourteen drakes (Anas Platyrhynchos) weighing 0.92 to 1.29 kg were bought from local market. They were also divided into two equal groups for experiment and control. They were treated in the same way as cocks. In order to show the toxicity feature (decreasing body weight), the dosage of gossypol was increased to 20 mg/kg after 9 weeks. At the end of 11th week all the drakes were sacrificed as the same way used for cock.

2. Measurement of LDH-X activities in cock, drake, mouse and rat

a. Preparation of samples

Spermatogenic cells were obtained from the following animals: male adult Spragre-Dawley rats (body weight ca 250-450g) and adult male Balb/cAn mice (body weight ca 20g) were obtained from the animal house of The Chinese University of Hong Kong; cocks (Gallus Domesticus) weighing about 1.5 kg and drakes (Anas Platyrhynchos) weighing about 2.3 kg were bought in local market. The rats and mice were

sacrificed by cervical dislocation, while the cocks and drakes were sacrificed by vasotomy in jugalar vessel. After all animals were sacrificed, the testis were removed and decapsulated. Blood capillaries were carefully removed. The seminiferous tubules were washed twice with small quantity of phosphate buffer saline (PBS) and were chopped into segments with a razor blade or with a pair of scissors. Approximately 8ml PBS were added to the teared samples and the suspension was transferred to a clean test tube where it was pipetted up and down with a Pasteur pipette for 20 times. This sample was then left to settle for 5 minutes. At the end of the waiting period, the tubules that sedimented to the bottom were discarded. The upper cell suspension that was free of tubules were centrifuged at 500g at 4°C for 15 minutes. The supernatant was discarded and the cell pellet was washed with PBS and centrifuged one more time in the same condition as the first time. The cell pellet resulted were resuspended in 5ml PBS. This cell suspension resulted was then sonicated with a ultrasonic cell disrupter, model W 200R, (Heat Systems-Ultrasonics, INC. U.S.A.) operated at 50% duty cycle for two minutes stopping at an interval of 20 seconds to prevent excessive heating. The samples were always put in an ice-cooled container to avoid the destoriation of enzymes by heat. The homogenate was centrifuged at 10,000g for 10 mins. at 4°C. Then the supernatant was used for enzyme assay.

b. Enzyme assay

The sonication preparation and enzyme assay procedure were adapted from those of Brooks (1978). Protein concentration was estimated by Lowry's method using BSA as the standard (1951). The enzyme assay was performed at 20°C against blanks in which the substrate was omitted in cells of 1cm light-path in a UV-240 spectrophotometer (Shimadzu corporation, Japan) by following the oxidation of nicotinamide coenzyme at 340 nm. The assay medium contained 100 mM Triethanolamine, 0.12 mM NADH, 10 mM α-ketovalerate and 2.5 μM rotenone with pH adjusted to 7.2 by HCl. The assay conditions were chosen to maximize activity and to ensure zero-order kinetics. The reaction rates were linear over the 2 minutes recording period and were proportional to the amount of spermatozoal homogenate. The assays were performed in triplicate and the mean values were taken. The enzyme activity was expressed in μmole of NADH oxidized per minute per mg protein.

III. RESULTS

A. Effect of Gossypol on the Spermatozoa of Cock and Drake

1. cock

Two of the experimental cocks died in the 6th and 7th week respectively. The other cocks of the experimental

group showed a 8-25% reduction of body weight in 11 weeks compared with that at the beginning of the experiment. However, their motility of sperm were greater than 80%. Two of them had smaller testes, weighing 6.5 and 9.1g respectively, while in the other two cocks', 14.0 and 26.4g. They were 14.0-29.1g in six control cocks. However, there was no difference in the morphology of germinal epithelia between the treated and the control groups, irrespective of the testicular size. The body weight of the control cocks was increased by 10-15%.

2. drake

At the 9th week, one drake of the experimental group died accidentally, and the body weight of the rest increased 4-9% compared with that at the begining of the experiment. At the 11th week their body weight decreased 4-29% respectively as compared with that at the 9th week. Semen containing motile sperm was collected once a week in three of the six remaining drakes, but could not be collected after the 10th week. When the drakes were sacrificed at the end of 11th week, the content of testes was small amount of sperm and their motility were less than 5%. The testes of gossypol group became smallar (0.2-1.6g) with light brown colour. In seven normal control drakes, although two of them had smaller testes weighing 0.3 and 0.7g, the testes of other five drakes weighed 2-17g, with light pink colour, which was quite different from that of

Type of Animal	Treatment	Body wt gain (%)	Colour of testis	Sperm motility(%)
Cock	Control (n=6)	↑ 10-15	light pink	> 90 ±10
	Gossypol (n=6)	↓ 8-25	light pink	> 80 ±12
Drake	Control (n=7)	↑ 13-17	light pink	> 90 ±15
	* Gossypol (n=7)	↓ 4-29	light brown	< 5 ±2

Table 2-1. The effect of gossypol in the sperm motility of cock and drake. 10mg/kg daily of pure gossypol (capsule) was administered to cocks and drakes of the experimental group for 11 weeks. The control animals were fed empty capsule alone. Number of animals was enclosed in parenthesis.
* The % decreased of the body weight was compared with that at the 9th week.

the gossypol-treated group. The body weight of the control group was increased by 13-17%. The above results were summarized in table 2-1.

B. The LDH-X Activities of Cock, Drake, Rat and Mouse

In order to obtain statistically significance, the number of each animal type used was: 20 mice, 10 rats, 6 cocks and 5 drakes. The specific activity of LDH-X from different kinds of animals was depicted in Fig. 2—1. It is shown that the specific activity of LDH—X in mouse was the highest and that in either cock or drake was just a half. These two species, cock, and drake, which was reported to contain no LDH-X in previous study have a clear cut higher activity than that of rat. The actual amount of LDH-X activity of these animals was shown in the legend of Fig 2-1 .

Specific activity of LDH X
(umoles of NADH oxidized/min./mg protein)
0.25
0.20
0.15
0.10
0.05
0.00
6*
10
6
5
Mouse
Rat
Cock
Drake

Fig. 2-1. Specific activity of LDH-X in different animal species. Number in column represents the number of animal used. Vertical bar on each column is the standard deviation. * In mouse, six sample were used. The testicular homogenate was collected by homogenizing the testis of three to four mice together to give enough material for one sample. The actual value of LDH-X specific activies of mouse, rat, cock and drake were 0.206 ±0.023, 0.061 ±0.009, 0.094 ±0.013, and 0.096 ±0.006 respectively. Values given were mean ±SD.

IV. DISCUSSION

LDH-X has been for a long time regarded as the target enzyme of gossypol. It was believed that the effect of gossypol on the testes was due to its selective inhibition on the sperm-specific enzyme LDH-X (Lee and Malling, 1981; Lee *et al.*, 1982). However, in our study, only the drake testes were sensitive to gossypol (the sperm motility was less than 5% after gossypol administration), while the cock testes were not (the sperm motility was greater than 80% after gossypol administration). Moreover, some authors had reported that gossypol did not significantly affect the motility of testicular and epididymal spermatozoa in mice (Shi and Zhang, 1980). Even with toxic doses, similar result was obtained (Hahn *et al.*, 1981). In our results, the level of LDH-X of mouse was very high. If LDH-X is the target enzyme of gossypol, the mouse LDH-X level should then be shifted due to its gossypol sensitivity and both the testes of cock and drake should be sensitive to gossypol (because LDH-X could be detected in the testes of these animals). Thus, the hypothesis of LDH-X being the target enzyme of gossypol antifertility action is worth suspected.

Furthermore, it has been suggested by the work of Eliasson and Virji (1983) recently that the effect of gossypol on sperm LDH-X activity is not the major action

for its antifertility action. Moreover, it was also found by Yu (1987) that there was no decrease in sperm LDH-X of the testis and the proximal segment of epididymis, while minor decrease in LDH-X was noted in the distal segment of epididymis, after the administration of gossypol for five weeks in rats. At the same time, the damage of spermatozoa and their spiral sheath mitochondria were observed only in the distal segment. By using histochemical observation to detect LDH-X, the spermatozoa of distal segment of gossypol-treated rat developed positive reaction much earlier than the control. The author concluded that the damage of the spermatozoa and mitochondria might be accompanied by subsequent leakage of LDH-X. It is unlikely that the antispermatogenic effect of gossypol is related to the inhibition of LDH-X. Therefore, it may be concluded that LDH-X is possibly not the target enzyme of gossypol. On the other hand, Tso suggested that although LDH-X may not be the target enzyme of gossypol antifertility, inhibition of LDH-X by gossypol might still be one of the many factors that acts together with other factors which cause infertility (Shi *et al.*, 1987).

In this study, both cock and drake revealed a definite LDH-X activity in their spermatogenic cells although the enzyme activity was less than that in mouse. However, more than twenty years ago Zinkham *et al.* reported that there was no LDH-X in duck, chicken, hog and cat testes when they

analysed LDH isomzymes in testicular homogenates from several animal species (Zinkham *et al*, 1964). The reason why Zinkham *et al.* could not detect LDH-X in cock, drake and hog testes might be due to the quantity of LDH-X was too small, or the starch gel they used for detection was not sensitive enough to do so. A further possibility is the lack of resolution of the gel electrophosis used in the experiment. Previously, Tso and Lee had detected LDH-X in boar while Zinkham *et al.* has clamined no such activity in a boar related species, hog. This result gives an additional support to our argument. It seems that sensitivity of the determination is an important factor for its identification before this work is done. It is believed that using cDNA probe to detect LDH-X is a feasible and convinicible way (Millan *et al.*, 1987; and Edwards *et al.*, 1987). Then, it is very interesting to identify the LDH-X activity in different species, which has been supposed to be absent.

CHAPTER 3 THE EFFECT OF GOSSYPOL ON THE METABOLISM OF ZINC IN TESTIS, HAIR AND RETINA OF HAMSTER AND MOUSE

I. INTRODUCTION

A. The Physiological Role of Zinc in Animals

Zinc (Zn) is an element known to be of importance for normal cell functions. It has been shown to participate in a wide range of enzymatic processes involving in many aspects of the intermediate metabolism and physiological functions of the organisms. Biochemically, it acts as catalyst or cofactor in enzyme system with roles ranging from relatively weak, non specific ion effects (metal-ion activated enzymes) to highly specific associations (metallo-enzymes) in which the metal is firmly attached to the protein in a fixed number of ions per molecule. As a typical example, the cytocuprein, containing a number of zinc, is an important moiety in superoxide dismutase which catalyzes the superoxide dismutation (this enzyme will be discussed in more detail in the next chapter).

Besides its contribution to enzymatic process, it has been shown that animals which consume zinc-deficient diets eventually develop a variety of pathologies including growth retardation, alopecia, dermatitis, anorexia, abnormal immune function, abnormal nitrogen metabolism, hypogeusia, impaired reproductive capacity, impaired

connective tissue metabolism and behaviourial defects. Actually, testicular atrophy and failure of spermatogenesis have been known to be the direct and conspicuous features of Zn deficiency in rats for more than 20 years (Mawson and Fischer, 1952). It is reasonable that a zinc deficiency generated by any cause including one that is affected by say gossypol administration will also manifest a testicular atrophy and failure of spermatogenesis.

B. The Relationship between Gossypol and Divalent Ions

Chemically, gossypol was reported to combine easily with a variety of divalent ions (Haas and Shirley, 1965; Abou-Donia, 1976). The most typical one is ferric iron. It has also been reported that gossypol could chelate with zinc to form an orange precipitate (Ramaswamy, 1968). More recently, gossypol was proposed to chelate with Mn^{2+} to form a 2:1 complex which induced a functional Mn^{2+} deficiency in the testis (White *et al.*, 1988). Historically, the toxicity of gossypol was found to be reduced or eliminated by adding $FeSO_4.7H_2O$ to the cottonseed cake which was fed to poultry (Withers, 1913). But when $FeSO_4.7H_2O$ was replaced by ZnO, such effect was not counteracted (Smith, 1970).

Our concern about the effects of gossypol on the zinc metabolism is based on the following considerations : (1) gossypol has an inhibitory effect on spermatogenesis

(National Coordinating Group for Male Contraception, 1978); (2) gossypol is a chelating agent which can combine with divalent ions. (3) zinc is an essential trace element for spermatogenesis (Amelar and Dubin, 1973). In spite of the many practical difficulties encountered in observing directly the change of zinc content in reproductive organs of males (Yu *et al.*, 1981), we would like to know, first of all, whether gossypol can affect zinc metabolism in the body.

Hair is one of the tissue types used for this investigation. It is a relatively permanent structure once it is formed. Its zinc content, once incorporated into the hair composition, will remain unchanged as the finished structure is expelled from the skin surface to become isolated from the body's continuing metabolic activities. The endogenous components of a hair reflect only those metabolic events that occurred during the relatively short time of its formation. Ordinarily, this period is as short as several days. Since trace element analysis of hair is precise, accurate and reproducible (Hopps, 1979), it is a suitable sample for estimating the body's trace element content. Retina is a structure reported to contain a high level of zinc (Schroeder, 1976). Therefore, in this chapter, besides testis and hair, retina was also chosen for studying.

II. MATERIALS AND METHODS

A. Reagents

All the reagents used were of analytical grade. Pure gossypol (content 100.6%, by spectrophotometry; melting point 172.4°C) was a gift from Yu (Shanghai Institute of Physiology, Academia Sinica). The following chemicals were purchased from Riedel-de Haen Chemical Co. (West Germany): sodium chloride; potassium dihydrogen phosphate; di-sodium hydrogen phosphate. 70% nitric acid was purchased from Kock-Light Laboratories Ltd (UK). Corn oil under the trade mark of Hop Hing Oil Factory Ltd, Hong Kong, was bought from local market. Zinc and iron atomic absorption standard solution and pentobarbital sodium salt were purchased from Sigma Chemical Co. (USA).

B. Treatment of Animals for the Zinc Content Study

Male Chinese Golden hamsters (ca 160-180g) and ICR mice (ca 38-50g) were obtained from the animal house of The Chinese University of Hong Kong. They were divided into two groups respectively. The drug group was force-fed with gossypol in corn oil of 30 mg/kg/day by gavage as described in appendix 1, while the control group with corn oil alone. Experimental animals had poor appetite after the treatment of such a large dose of gossypol. In order to keep pace

with such a reduction in gained body weight in the experimental animals, the control animals had to be fed with reduced but calculated amount of food to even out any difference in weight gained between the groups. All the animals were raised under standardized laboratory conditions in an temperature controlled animal room with lights on from 0600 to 2100 hours. In order to eliminate the other factors which could affect the effect of gossypol (which had been mentioned in appendix 1), the lower protein content diet, Laboratory Rabbit Chow was employed instead of the Rodent Laboratory Chow. The body weights of all control and gossypol-treated animals were measured and recorded once a week during the experimental period. The feeding dose was readjusted according to the body weight weekly.

1. Detection of zinc content in hamster hair

Eighty hamsters were used. Gossypol was force-fed for a period of 10 to 60 days without a day break. At different time intervals (10, 20, 30, and 60 days after the starting of gossypol treatment), the hamsters were sacrificed by anesthelization with excess dose of pentobarbital by an intraperitoneal injection. The back hair was cut out with a pair of stainless scissors for Zn and Fe determination. In order to prevent any contamination, the stainless scissors was cleaned with deionized water before use every time. At the same time, semen was taken from the vas

deferens and the sperm motility was checked by direct microscopic observation.

2. Detection of zinc distribution in the hair, testes and retina of hamsters and mice

Thirty hamsters and mice were used. Since the iron content was decreased after 20 days of gossypol administration, the duration of gossypol feeding was 30 days in this study. At the end of treatment, the mice were sacrificed by cervical dislocation, while the hamsters were anesthelized with excess dose of pentobarbital by intraperitoneal injection. After the animals were sacrificed, semen was taken out from the vas deferens and the sperm motility was observed microscopically. Then the testes, eyeballs and the hair along the backside region, of each animal were taken out. The removed testes were homogenized in 5 volume (ml/g tissue) of phosphate buffer saline (PBS) at pH 7, in an ice-cooled potter-Elvehjem homogenizer operated at full speed. The preparation was lyophilized accordingly. The removed eyeballs were placed in PBS. Extraneous fat and muscle tissue was removed from the outer surface of the eyeball which was then hemisected, and the vitreous humour was carefully removed. Retina was sliced out by a dissection probe. Then the retina sample was homogenized in 1ml PBS and lyophilized in the same condition as that used in obtaining testis sample. Before being lyophilized, about 0.1ml sample was taken out for

protein determination by Lowry's method using BSA as the standard. The lyophilized testis samples and the hair were weighed for zinc and iron assay. Because of the small amount of lyophilized retina, the whole sample was used for zinc and iron determination.

C. Determination of Zinc and Iron

The following method was adapted from Yu *et al.* (1979). Suitable amount of hair and tissues obtained from the test animals were weighed in test tubes. In order not to alter or contaminate the hair, no cleaning or washing procedure was used (Zhang and Yu, 1989). To each sample, 0.5ml of 70% nitric acid was added and the content was heated over 100°C in a waterbath for 25-45 minutes to digest all solid material. Deionized water was used when dilution was needed. Zinc and iron contents were detected by atomic absorption spectrophotometry (Spectr AA-10, Varian Techtron Pty. Ltd., Austratia) according to the standard solutions calibrated.

III. RESULTS

A. Amount of Zinc in Hamster's Hair

Although there was no difference in the zinc content between the gossypol-treated hamster and control, a marked

decrease in iron content after 20 days of gossypol feeding was observed. The difference between the highest and the lowest content of iron was about 50 µg/g. The zinc content was between 180-210 µg/g dry weight. This was depicted in Figure 3-1. Since the same animal was tested for both element content, in order to compare the gossypol effect on this element, a new value by dividing the ratio of iron/zinc of the gossypol treated-group and that of the of the control was obtained, as shown in Fig. 3-2. Basically in the beginning 20 days, the quotient was practically not affected, being close to one. However, there was a drastic decrease after 20 days of gossypol treatment and the quotient was stabilized to a new value in about one month (Fig. 3-2). This value is about 0.5.

During this experiment, some hamsters were found eye-bleeding and finally became blind. Nevertheless, retina was a rich source of zinc (Schroeder, 1976). In order to see whether there was any change in zinc content in other organs after gossypol feeding, another experiment to detect the distribution of zinc content in hair, testis and retina of gossypol-treated hamsters and mice was taken.

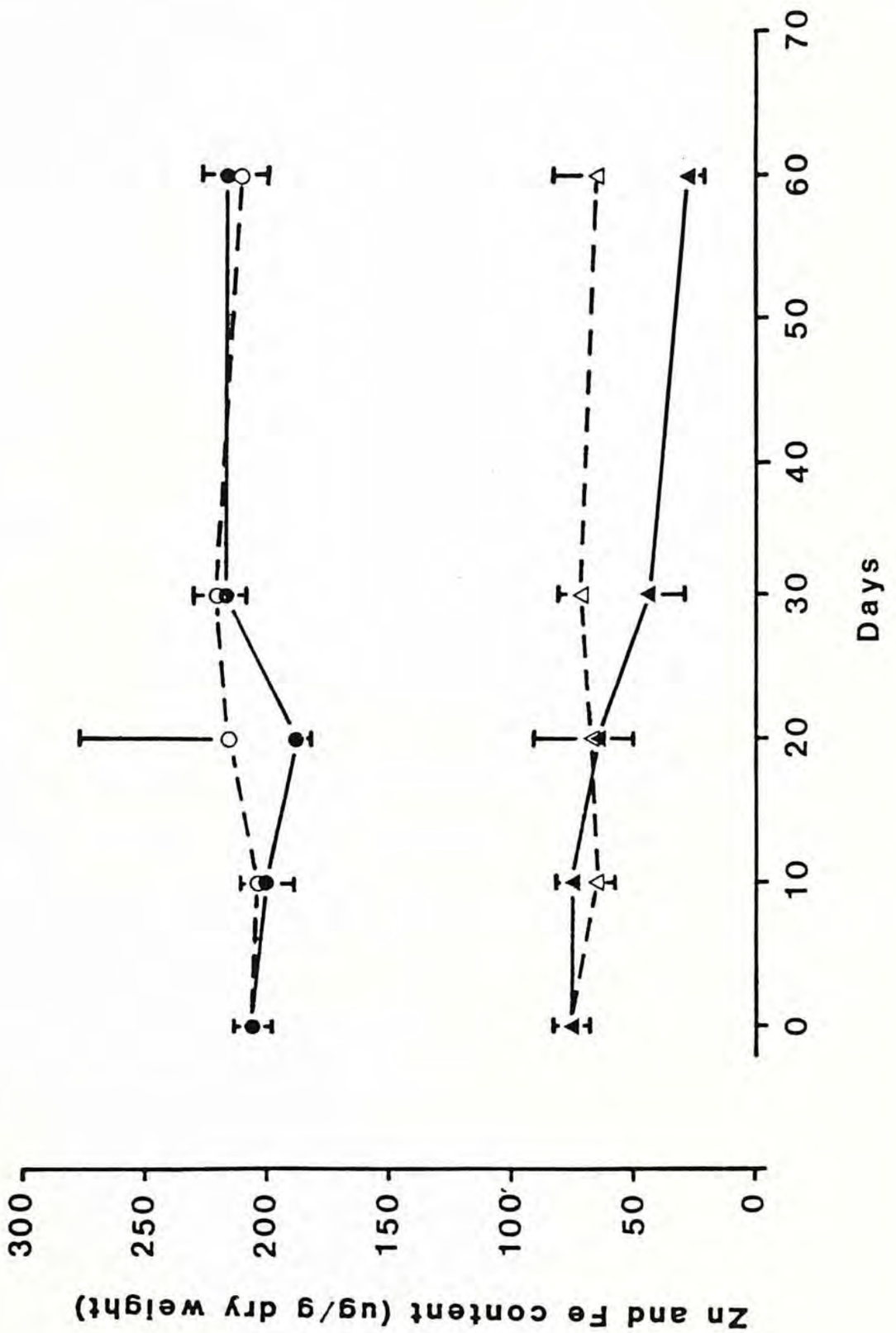
Zn and Fe content (ug/g dry weight)
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Days

Fig. 3-1. Effect of gossypol on the Zn and Fe levels in the hair of gossypol-treated hamsters. ● and ○ represent the zinc content in the gossypol-treated and the control animals correspondingly. The iron level in treated and control hamsters were depicted by ▲ and △ correspondingly.

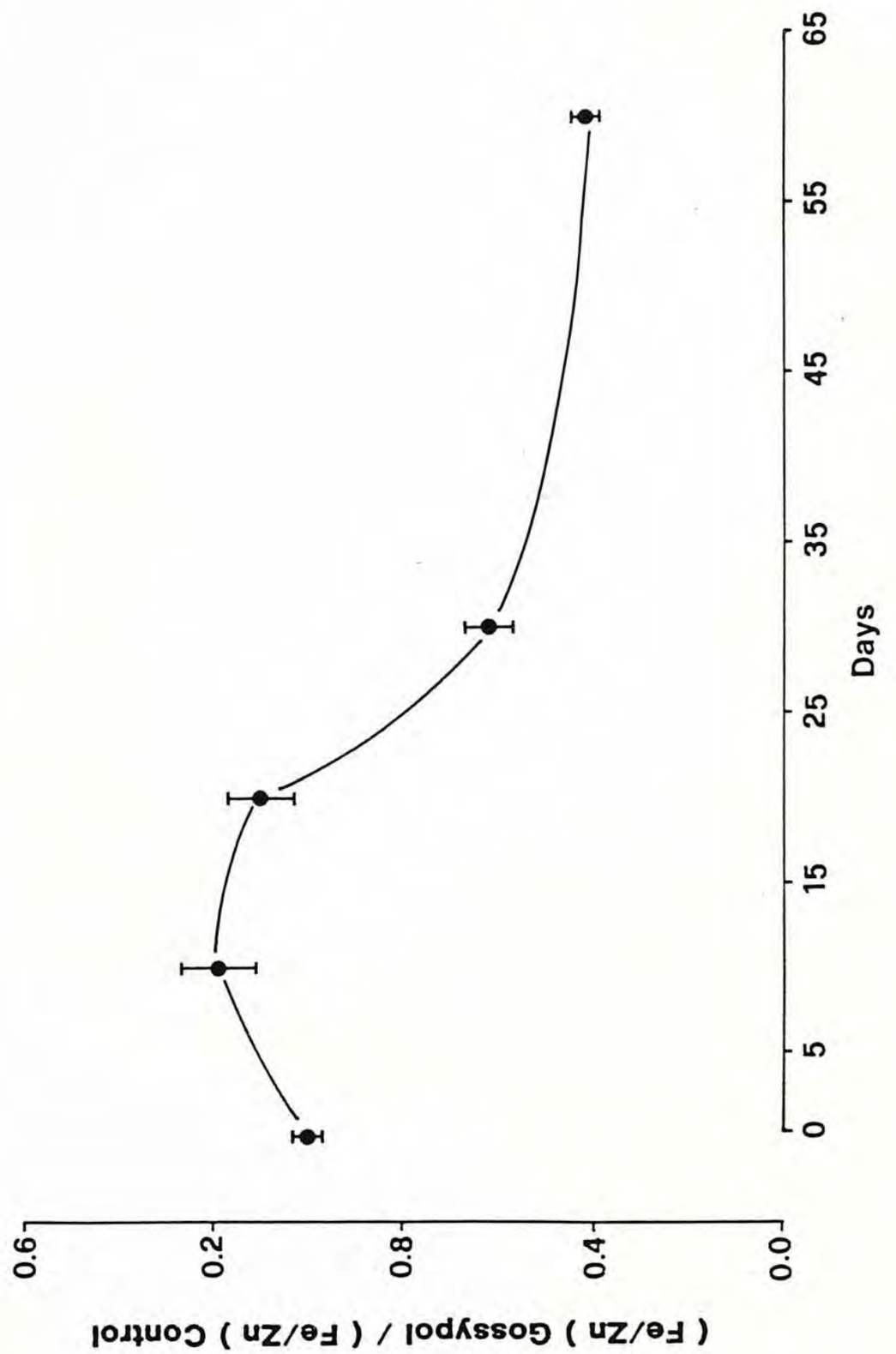
(Fe/Zn) Gossypol / (Fe/Zn) Control
0.0
0.4
0.8
0.2
0.6
0
5
15
25
35
45
55
65
Days

Fig. 3-2. Ratio of (Fe/Zn) gossypol and (Fe/Zn) control of hamster hair. 30 mg/kg/day of pure gossypol was force-fed orally each day for 10 to 60 days.

B. The Distribution of Zinc Content in Hair, Testis and Retina of Hamster and Mouse

There was a significant increase in the content of zinc and iron in mice and hamsters retina which had been force-fed with gossypol. Although the zinc contents in hamster's hair and testis were both decreased, it was not statistically significant in hair. The zinc contents of mice hair and testis were both increased insignificantly. Considering the iron content, it has decreased in hamster hair but increased in testis. They were both statistically significant. In mice, the pattern was different. It was not only decreased in hair but also in testis. However, it was only statistically significant in hair. These results were summarized in table 3-1.

Sample		Hamster			Mouse		
		control (n=10)	gossypol (n=8)	difference (%)	control (n=10)	gossypol (n=9)	difference (%)
Hair	Zn	239.2 ±23.5	225.2 ±19.6	-5.8	134.0 ±9.0	138.8 ±9.2	+3.6
	Fe	62.6 ±8.2	51.9 ±6.0	-17.0 *	41.0 ±9.4	27.6 ±7.4	-32.6 *
Testis	Zn	133.7 ±4.0	120.4 ±18.3	-9.9	114.0 ±14.6	117.8 ±9.9	+3.3
	Fe	67.1 ±5.7	77.8 ±6.1	+16 *	166.6 ±42.0	134.1 ±12.3	-19.5 *
Retina	Zn	0.1 ±0.0	0.2 ±0.0	+80 **	0.2 ±0.1	0.8 ±0.5	+229 *
	Fe	1.9 ±1.0	12.3 ±4.3	+552 *	1.2 ±0.8	5.0 ±2.0	+334.8 **

Table 3-1. The Zn and Fe content in various tissues of hamster and mouse. For the treated animals, 30 mg/kg/day of pure gossypol was used for 30 days uninterrupted. Each value represents mg/g dry weight ±SD. Number of animals was indicated in parenthesis. *$p<0.05$, **$p<0.001$ compared with control. During the experiment two experimental hamsters and one experimental mouse died probably due to high toxicity. The unit used in retina was mg/mg protein sine the small amount of retina sample collected.

IV DISCUSSION

The finding in the hamster's hair suggested that gossypol did not affect zinc metabolism in hair. But it decreased the iron level after 20 days of gossypol feeding (Fig. 3-1). It means that gossypol could interact with iron, which is coincident with the results of previous studies (Jonasson and Demint, 1955; Bintrago *et al.*, 1970; Herman and Smith, 1973; and Skutches *et al.*, 1973). From Figure 3-2, we can see that the change in zinc and iron content in hair of gossypol-treated animals are neither proportional nor reversibly proportional to each other. This indicates that the effect of gossypol on iron does not correlate to the effect of gossypol to zinc.

Furthermore, it was also confirmed that the zinc level in hair and testis of hamsters and mice is not influenced by gossypol although a species difference was demonstrated (Table 3-1). For example, it was decreased in hamster hair and testis but was increased in those of mice. The reasons for this species difference in the increase or decrease in zinc level await further studies. This phenomenon was also showed in the iron content determination. The previous reports that discussed this feature was very confusing (Stutches *et al.*, 1973 and 1974).

However, a marked increase in zinc content of mice and

hamsters retina was observed (Table 3-1). It might be said that gossypol could affect the zinc metabolism in retina, although this change might not necessarily have any relationship with the antifertility mechanism of gossypol. An increase of zinc in this region after gossypol force-feeding might be resulted from chelation of zinc ion by gossypol to form complex which was not excreted in time. This feature had also been reported by Skutches *et al.* (1973, 1974) who described the increased iron content in swine liver after treating with gossypol. So if we take gossypol for a long time and at a high dose, it will affect the zinc metabolism in retina at last.

Apparently, the zinc depletion phenomenon was a cause only at high gossypol intake. Even in clinical study, no such report has been reported, and there is no indication that the gossypol effect on zinc depletion is an accumulated one. A detail study on this picture is required to improve the efficacy of gossypol as an contraceptive means.

CHAPTER 4 EFFECT OF GOSSYPOL ON THE ANTIOXIDANT DEFENSE SYSTEM IN THE TESTIS OF HAMSTER

I. INTRODUCTION

In the course to find out the antifertility mechanism of gossypol, different biochemical changes in the male reproductive system have been investigated. Among them, the inhibitory effect of gossypol on various enzyme activities have been studied extensively. Moreover, the enzyme systems chosen for these studies, either *in vivo* or *in vitro*, were essentially related to energy metabolism. These studies, however, in spite of the great effort exerted, have not succeeded in identifying a target enzyme. Lactate Dehydrogenase-X, an enzyme that plays a unique and key role in sperm physiology was once considered to be a putative target enzyme. But more and more evidences from gossypol structure, antifertility study and other investigations obtained from this laboratory as well as from other groups have altered this model (Kalla and Wei, 1981; Lee and Malling, 1981; Tso and Lee, 1982a; Tso and Lee, 1982b; Kennedy *et al.*, 1983; Olgiati *et al.*, 1984; and Shi and Friend, 1986). A recent *in vitro* study examining human sperm and rat liver microsomes found that gossypol promoted the formation of reactive species of oxygen such as hydrogen peroxide and superoxide radicals. The authors suggested that the common mechanism that underlies all of the toxic effects might be gossypol is related to free

radical injury (Peyster *et al.*, 1984). Later, it was also confirmed by Wu and Yu (1986) that gossypol promotes the formation of oxygen radicals when incubated with rat liver and kidney microsomes. Unfortunately, the only detoxification enzymes so far studied is the Glutathione-S-transferase. It is an enzyme that functions in detoxication and has been reported to be inhibited by gossypol. But further study has been abandoned for unknown reasons (Maugh, 1981).

Reactive species of oxygen are implicated as a cause of a multitude of pathologic changes. It has been suspected that an interaction of the free radical with either a protein or a lipid moiety often changes the molecular configuration in such an extend that it severely hampers normal cell functions. This interaction quite often lead to the death of a cell (Freeman, 1982). The interaction of free radicals with DNA, the genetic material of the cell, may also cause a damage to the integrity of the greater macromolecular strand breakage. Cells often die after this form of damage (Halliwell, 1985). Those that survive may undergo mutations. Cell death caused by reactive species of oxygen is likely to be a combination of an overwhelming production of free radicals and an ineffective antioxidant defense system.

The present study examines the effects of orally

administrated gossypol acetic acid on the antioxidant defense enzymes taken from hamster testis. At the same time, intratesticular injection of some scavengers is studied to see whether they can eliminate the effect of gossypol or not and malonaldehyde (MDA), the product of lipid peroxidation, is also examined to identify indirectly whether gossypol can produce free radicals which trigger lipid peroxidation.

A. Oxygen Radicals in Living Cells

Cells living under aerobic condition gain their bioenergy from the reduction of molecular oxygen to water and are inevitably prone to oxygen toxicity, as highly reactive intermediates, such as superoxide anion radical (O_2-.), hydrogen peroxide (H_2O_2), singlet oxygen (O_2·), or hydroxyl radical (OH.), will be formed during the reduction of oxygen (Boveris *et al.*, 1972; Boveris and Chance, 1973; and Loschen *et al.*, 1974). Though most of the oxygen consumed by respiring cells is used by the cytochrome oxidase system which accomplishes the tetravalent reduction of oxygen to water without the release of any free reactive intermediates, both O_2-. and H_2O_2 are still produced as normal minor metabolites within the cells by a variety of enzymes. In rat liver up to 5% of the oxygen is consumed for the production of H_2O_2 (Boveris *et al.*, 1972; Boveris and Chance, 1973). A remarkable multiplicity of sub-

cellular sources of these intermediates is found. For example, H_2O_2 is produced within the peroxisomal, microsomal, mitochondrial, and soluble fractions of the cells, superoxide anion is generated by flavin enzymes and iron-sulfur protein in the cytosol and by the auto-oxidation of reduced ubiquinone in the mitochondria (Massey *et al.*, 1969; Misra and Firdovich, 1972; Fridovich, 1974a,b; Loschen, 1975; and Cadenas *et al.*, 1977). The pronounced reactivity of H_2O_2, and O_2-. provides the basis of many important biochemical reaction (Serif and Kikwood, 1958; Mead and Lewis, 1963; Hasting, 1966; Oshino *et al.*, 1973; and Klebanoff, 1974).

Though being present essentially in low concentration, these highly reactive intermediates of oxygen reduction if not maintained at a steady level can directly or indirectly cause substantial damage to living cells (Halliwell, 1974; Cohen and Heikkila, 1974). The simultaneous existence of superoxide anion and hydrogen peroxide favours the formation of the additional oxygen species, hydroxyl radicals and singlet oxygen, which in turn may attack a variety of organic compounds and lead to organic and lipid peroxide formation (Haber and Weiss, 1934; Arneson, 1970; Cohen and Heikkila, 1974; Chance *et al.*, 1978; and Tappel, 1978).

B. Oxygen Damage of Spermatozoa

Spermatozoa have limited life span under aerobic conditions. Seminal plasma or extraneous spermatozoa are not capable of retaining their motility by endogenous respiration even in the absence of these substances. Instead, it has long been considered as the result of a toxic effect of oxygen. Thus, oxygen represents a potential hazard to sperm structure of function (Mana and Lutwak-Mann, 1975).

The oxygen free radicals can induce lipid peroxidation and cause damage to cellular membranes (Fridovich, 1974 and 1978). It was also reported that mammalian spermatozoa are sensitive to lipid peroxidation, and the peroxides produced from the unsaturated fatty acids of the sperm phospholipids are strongly spermicidal (Jones and Mann, 1973; Jones and Mann, 1976; Jones *et al.*, 1978; and Jones *et al.*, 1979).

C. Antioxidant Defense System

In order to protect the fluidity and structural integrity of plasma membrane, an array of defense mechanisms is set up. Two types of antioxidants are known: the preventive antioxidants such as superoxide dismutase, catalase and peroxidase and the chain-breaking antioxidants scavenge radicals to terminate free radical reactions and prevent chain propagation reactions.

1. Enzymatic antioxidants

Superoxide dismutase, in conjunction with glutathione peroxidase and catalase, set up the defense mechanism against the oxidative stress (Halliwell, 1974). Superoxide dismutase eliminate the superoxide radical in the first line of defense. Subsequently, the hydrogen peroxide is removed by catalase and/or glutathione peroxidase. If the reactive intermediates escape both defenses and cause peroxidation of unsaturated membrane fatty acid, glutathione peroxidase can act as the last line of defense to prevent further propagation of the radical chain reaction that leads to lipid peroxidation, deterioration of membrane lipids and severe impairment of energy related membrane functions (Wendel, 1980). These reactions are summarized in Fig. 4-1.

2. Non-enzymatic antioxidants

Tissues also have a variety of nonenzymatic antioxidants for preventing from the damage by free radicals. Vitamin E (a series of isomers of tocopherol) is a lipid phase antioxidant that partitions into all membranes and converts superoxide anion, hydroxyl, and lipid peroxyl radicals to less reactive forms. It acts by donating a hydrogen ion to the radical and thereby confining the effect of the radical; in addition, a stable vitamin E radical is formed. Vitamin C is an aqueous phase

Fig. 4-1. Diagram of enzyme systems to bypass reactive intermediates being released during sequential univalent reduction of molecular oxygen.

Fig. 4-2. A proposed scheme by which ascorbate metabolism participates in detoxification of OH·

antioxidant. The possibility is currently considered that ascorbate scavenges OH• resulting in formation of the ascorbate free radical (AFR). Pairs of AFR spontaneously disproportionate to form ascorbate and dehydroascorbic acid (DHA), the latter being a toxic molecule that is normally maintained at a low concentration in plasma. Dehydroascorbic acid spontaneously degrades to diketogulonic acid, which is devoid of antiscorbutic or other metabolic activity. A direct and energetically efficient pathway for reforming the nontoxic and useful ascorbate would be through direct reduction of AFR by one-electron transfer. This procedure is depicted at Fig. 4-2. Selenium is a component of glutathione peroxidase (GSH-Px) which catalyzes the destruction of H_2O_2, lipid hydroperoxides (ROOH), and other hydroperoxides. The presence of selenium in GSH-Px, which decomposes lipid peroxides, helped to explain many of the nutritional effects of selenium as an apparent antioxidant.

II. MATERIALS AND METHODS

A. Reagents

All chemical reagents used in these experiments were of analytical grade. The following reagents were purchased from Sigma Chemical Co. (U.S.A.): glutathione reduced form; beta-NADPH (tetrasodium salt); EDTA (tetrasodium salt);

sodium azide; glutathione reductase from Bakers yeast; hydrogen peroxide; 1-chloro-2,4-dinitrobenzene (CDNB); epinephrine; potassium cyanide; superoxide dismutase from bovine erythrocytes; copper sulfate; potassium sodium tartrate; bovine serum albumin (BSA); sodium selenite; pure gossypol; 97% 1,1,3,3-tetraethoxypropane; Sodium dodecyl sulfate and thiobarbituric acid. Sodium carbonate; dipotassium hydrogen phosphate; sodium hydroxide; Folin-Ciocalteus reagent; L-ascorbic acid and phosphotungstic acid were purchased from Merck Chemical Co. (West Germany). Potassium dihydrogen orthophosphate was purchased from BDH Chemical Ltd. (U.K.). Sodium dihydrogen phosphate-2-hydrate; ethanol absolute; sodium chloride; disodium hydrogen phosphate-12-hydrate and butanol were purchased from Riedel-de-Haen Chemical Co. (West Germany). Anaesthetic ether was purchased from May and Baker Ltd. (U.K.). Vitamin E (97%) was purchased from Aldrich Chemical Co. Ltd. (U.K.) Gossypol acetic acid (99.9% pure) was provided by the special Programme of Research in Human Reproduction World Health Organization, Geneva, Switzerland. Pure (-)-gossypol was a gift from The Institute of Materia Medica of the Chinese Academy of Medical Sciences, Peoples Republic of China. The purity of gossypol is above 99%, $[\alpha]_D^{20}$ =-362.9. Corn oil (Lion and Globe), a product from Hop Hing Oil Factory Ltd., Hong Kong was purchased from local market.

B. Experimental Animals

Male Chinese Golden hamsters (170-250g) were obtained from the animal house of The Chinese University of Hong Kong. They were raised under standardized laboratory conditions in air-conditioned animal room with lights on from 0600 to 2100 hour. The animals had free access to water and commercial rat rations (Rodent Laboratory Chow #5001, Labchows, Purina Mills, Inc.).

C. The effect of scavengers on the antifertility of gossypol

In these experiments, vitamin C, vitamin E and selenium were used as the scavengers. They were injected into the hamsters testis by intratesticular method. This method was taken by injection into the testis over the skin of scrotum after fixing the testis in the scrotum by another hand (Zhang and Tso, in press). The antifertility effect of gossypol were determined by the presence of immotile sperm in vas deferens. The sperm motility was observed and recorded as that described in appendix 1. Gossypol stock solution was prepared by dissolving 4mg of pure (-)-gossypol in 1ml corn oil. In order to avoid the possible immediate reaction between gossypol and scavengers, injection was carried out 24 hours before the introduction of the scavenger by the same technique.

1. Vitamin C

Twenty-four hamsters were divided into six equal groups. Fixed concentration of gossypol (0.2mg per testis) and different concentration of vitamin C were injected into the testis. Firstly, 0.05ml of stock gossypol solution was injected into the right side testis of all group's hamsters, while equal amount of corn oil was injected into the left side testis. After 24 hours, except the first group which acted as the control, the other five group's hamsters were injected with 0.05ml of different concentrations of vitamin C respectively in both left and right side testis, so that each testis accepted 10mg, 7.5mg, 5mg, 2.5mg and 1.25mg respectively in the five groups. All the hamsters were sacrificed by aspiration of excess anaesthetic ether at 14 days after the treatment was started. The sperm activity was observed under microscope. The injected 10mg, 5mg, 2.5mg vitamin C testes were taken for malonaldehyde (MDA) determination.

2. Selenium

The number of hamsters used and the procedure were the same as those used in vitamin C. In order to minimize the toxicity of selenium, only the right side testis was injected, while the left side testis was injected with 0.05ml distilled water. The stock solution of selenium was prepared by dissolving sodium selenite in distilled water. Therefore, the different concentrations of sodium selenite

accepted were 0.4mg, 0.3mg, 0.2mg, 0.1mg and 0.05mg. The testes were removed for MDA determination.

3. Vitamin E

Three groups of hamster were used. Each group contained four hamsters. 0ml, 0.05ml and 0.1ml of 97% vitamin E were injected into left and right side testes of three different group's hamsters respectively after gossypol injection. The procedure was the same as that used in the determination of the effect of vitamin C. But no testis was taken for MDA determination.

D. Chemical Interaction of Gossypol with Vitamin C and Sodium Selenite

In vitro study was taken. Pure gossypol was dissolved in 0.1 N NaOH solution first and then neutralized with 0.1 N HCl to pH 7. Vitamin C or sodium selenite was dissolved in distilled water and also neutralized with HCl or NaOH to pH 7. The spectrophotometric absorbance profiles of gossypol, vitamin C, gossypol+vitamin C (immediately and after 10 hours), sodium selenite and gossypol+sodium selenite (immediately and after 10 hours) were determined by UV-240 spectrophotometer. The concentrations of vitamin C, sodium selenite and gossypol in the cuvette were 5.7×10^{-5} M, 2×10^{-4} M and 6.4×10^{-6} M respectively.

E. Malonaldehyde (MDA) Assay

This method was adapted from Gower *et al.* (1987). The removed testis was homogenized in nine volume (w/v) of ice-cold phosphate buffer saline at pH 7, in a cooled potter-Elvehjem homogenizer in full speed. 0.5ml homogenate was mixed with 0.35% of SDS; 0.05 M of HCl; 0.75% of phosphotungstic acid and 0.165% of thiobarbituric acid immediately. The mixture was heated at 95°C for one hour. Final volume for incubation was 2ml. After cooling, 2.5ml butanol was added to the mixture for extraction.
The extracted supernatant was determinated at 532 nm and calculated according to the standard curve. The standard curve was determined by diluting 1,1,3,3-tetraethoxypropane in various concentrations and repeated the above procedure. The unit of MDA was nmol/ml.

F. The Effect of Gossypol on the Antioxidant Defense Enzymes

1. Drug treatment

12 hamsters were assigned randomly to two equal groups. The experimental group was force-fed with 10 mg/kg/day (6 days per week) gossypol in corn oil by gavage while the control group was fed with corn oil alone. Each hamster was weighed weekly and the dosage was adjusted according to the body weight. At the end of the 4th and the

5th week after the treatment was started, three experimental and three control animals were sacrificed respectively by aspiration of excess anaesthetic ether.

2. Preparation of samples for enzyme determination

After the hamsters were sacrificed, semen was taken out from the vas deference and sperm motility was observed under microscope. The testis was removed and decapsulated. Following the careful removal of blood capillaries, the testis were homogenized in five volumes (w/v) of ice cold phosphate buffer saline (PBS) at pH 7, in a cooled potter-Elvehjem homogenizer in maximum speed. The homogenate was centrifuged at different speed and time at 4°C for corresponding enzyme assay that followed. The centrifugation speed of Se-GSH-Px, glutathione transferase, superoxide dismutase, catalase were 10,000g for 30 minutes, 10,000g for one hour, 700g for 5-10 minutes, 20,000g for 30 minutes respectively. In order to increase the observable catalase level, suitable amount of absolute ethanol was added to the supernatant for catalase determination to a final concentration of 0.17 M. After it had been incubated in ice-bath for 30 minutes, 10% Triton X-100 at pH 7.4 was added to give a 1% final concentration (Cohen *et al.*, 1970).

3. Enzyme Assays

There were four enzyme assays being studied. They were

Se-GSH-Px, glutathione transferase, catalase, and superoxide dismutase. Different enzyme assay methods were described in the following. The protein concentration was estimated by Lowry's method (1951) using bovine serum albumin as the standard.

a. Se-GSH-Px [EC 1.11.1.9]

The method was adapted from that of Paglaia and Valentine (1967). The enzyme was assayed at 30°C in a 1.305ml reaction mixture containing 2.0 mM GSH, 0.1 mM NADPH, 100 mM K-phosphate buffer (pH 7.0), 3.0 mM EDTA, 1.0 mM sodium azide and 1 U glutathione reductase. The reaction was initiated after a 2-minute equilibration of the reaction mixture at 30°C by adding 0.12 mM H_2O_2. Absorbance at 340 nm was recorded with a UV-240 spectrophotometer (Shimadzu Corporation, Japan). The initial linear decrease in absorbance was used to calculate the initial reaction rate. The assays were performed in duplicate, alternating between control and gossypol-treated samples. Enzyme activity was expressed as µmole of NADPH oxidized per minute per mg protein.

b. Glutathione-S-Transferase [EC 2.5.1.18]

Enzyme activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). This method was adapted from that of Mannerick (1981). To

a 1-ml cuvette 10 mM sodium phosphate buffer (pH 6.5), 1 mM GSH, suitable amount of enzyme and deionized water were added to a final volume of 1ml. The reaction, which was carried out at 30°C, was started by addition of 0.95 mM CDNB in 95% ethanol. The reaction was monitored spectrophotometrically by the increase in absorbance at 340 nm. A correction for the spontaneous reaction was made by measuring and subtracting the rate in the absence of enzyme. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 µmole of S-2,4-dinitrophenylglutathione per minute at 30°C using 1 mM of GSH and CDNB. Specific activity was expressed as units per milligram of protein.

c. Catalase [EC 1.11.1.6]

Catalase activity was measured by the breakdown of H_2O_2:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

The method used was based on that of Aebi (1983). Suitable amount of homogenate was added to a 10.5 mM solution of H_2O_2 in 50 mM phosphate buffer (pH 7.0) at 25°C. The final volume of assay was 1ml. The degradation of H_2O_2 was monitored by measuring the decrease in absorbance at 240 nm since H_2O_2 gave high absorbance at this wavelength. One unit of catalase activity was defined as those decomposing 1 µmole of H_2O_2 per minute.

To verify that the activity measured was that of catalase, a control containing sodium azide (NaN_3), an inhibitor of catalase, in a final concentration of 1 mM was used (Theorell and Ehreaberg, 1952; Cederbaum, 1987). The difference between the activities with and without azide was interpreted as the actual activity.

d. Superoxide Dismutase (SOD) [EC 1.15.1.1]

SOD is a family of metalloenzymes which is known to accelerate the spontaneous dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen. The assay was based on the ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 (Misra and Fridovich, 1972). The production of adrenochrome in a final volume of 1ml containing 0.3 mM epinephrine, 0.1 mM EDTA, and 50 mM sodium carbonate buffer (pH 10.2) at 30°C was followed at 480 nm. One unit of enzyme was the amount of SOD capable of inhibiting 50% the rate of epinephrine oxidation observed in the control in which no enzyme was added. The activity measured was total activity of SOD.

III. RESULTS

A. The Effect of Scavengers on the Antifertility of Gossypol

The % of sperm motility recovered from gossypol

injection by vitamin C and sodium selenite was concentration dependent. The higher the concentrations of vitamin C and sodium selenite, the more the % of sperm motility recovered (Fig. 4-3, 4-4). Up to 10mg per testis, vitamin C could completely recover the % of sperm motility (Fig. 4-3). This phenomenon could also be observed when 0.4mg of sodium selenium was employed (Fig. 4-4). However, the above feature could not be shown in vitamin E administration even when the amount has increased to 0.1ml that was the highest acceptable amount the testis could maintain (Table 4-1).

B. Chemical interaction of Gossypol with Vitamin C and Sodium selenite

The spectrophotometric absorbance profiles of gossypol, vitamin C, gossypol+vitamin C (immediately and after 10 hours), sodium selenite, and gossypol+sodium selenite (immediately and after 10 hours) were depicted in Fig 4-5 and 4-6. The gossypol+vitamin C sample showed an additive profile of the gossypol sample and vitamin C sample instead of a shifted profile compared with the latters. A typical example was one of the peaks of the profile of gossypol between 400 and 300 nm also occurred at the profile of gossypol+vitamin C. The overlap peak between 300 and 190 nm of gossypol and vitamin C were added together at the profile of gossypol+vitamin C (Fig. 4-5a,

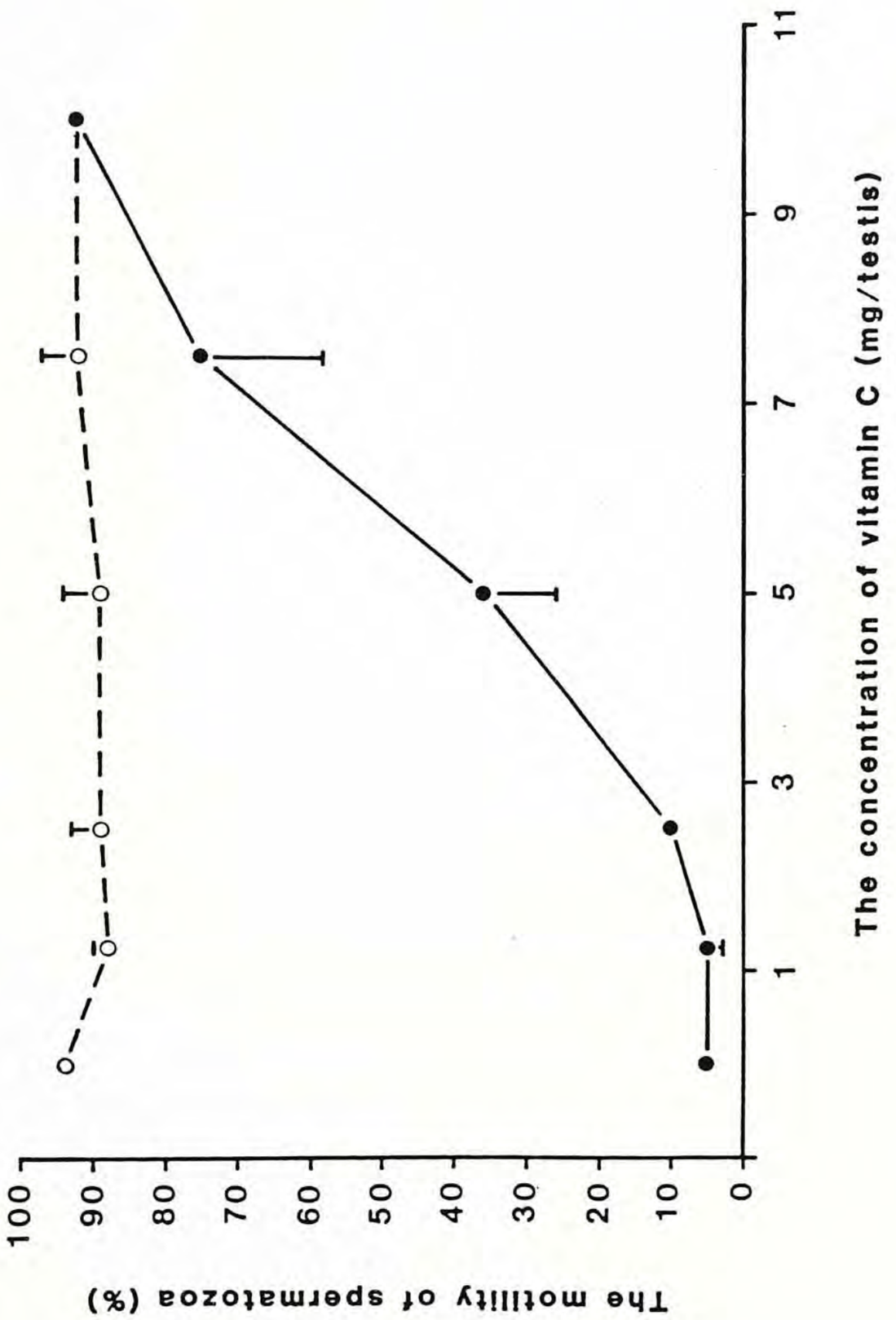
The motility of spermatozoa (%)
100
90
80
70
60
50
40
30
20
10
0
1
3
5
7
9
11
The concentration of vitamin C (mg/testis)

Fig. 4-3. Effect of vitamin C in compensation for the antifertility effect of gossypol. Each bar represents a total of 4 hamsters in which one testis received oil and vitamin C as control while the remaining testis received a single dose of (-)- gossypol (200µg/testis) followed by vitamin C 24 hours afterward. Sperm from vas deferent were collected from sacrificed hamster at 14 days after treatment. The open circle represents the control and the close circle represents the fixed amount of gossypol but varying amount of vitamin C.

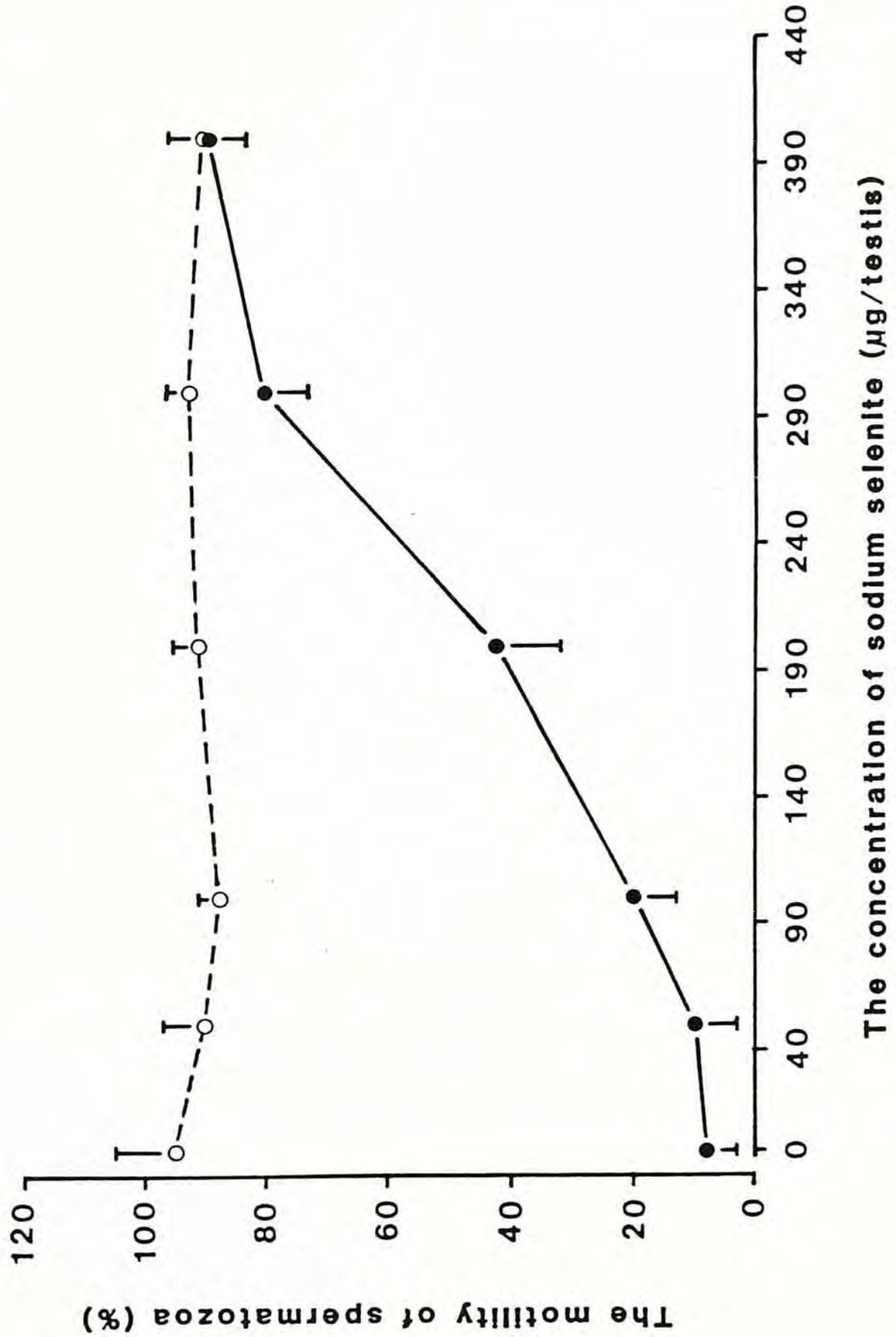
The motility of spermatozoa (%)
120
100
80
60
40
20
0
0
40
90
140
190
240
290
340
390
440
The concentration of sodium selenite (μg/testis)

Fig. 4-4. Effect of sodium selenite in compensation for the antifertility of gossypol. The number of hamster and the procedure was the same as that using in Fig. 4-3 but the control received distilled water instead of sodium selenite in order to prevent the toxicity of selenium. The open circle represents the control and the close circle represents the fixed amount of gossypol but varying amount of sodium selenite.

Group number	Amount of Vitamin E used (ml/testis)	Motility of spermatozoa (%)	
		Control (Vit E.+ oil)	Experiment (Vit E.+ gossypol)
I (4)	0	97.0 ± 1.3	6.9 ± 2.4
II (3)	0.05	90.0 ± 13.0	< 5.0 ± 3.0
III (4)	0.1	92.0 ± 11.0	< 5.0 ± 4.0

Table 4-1 The relationship between the antifertility effect of gossypol and vitamin E injected. (-)-gossypol (200μg/testis) and different amount of 97% vitamin E solutions were injected intratesticularly into the hamsters' testes at day 0, the result was developed at the end of 14 days. The percentage motility of spermatozoa in vas deferens was represented by mean ± S.D.. Number of animals was enclosed in parentheses. During the experiment, one hamster died in group II with unknown reason.

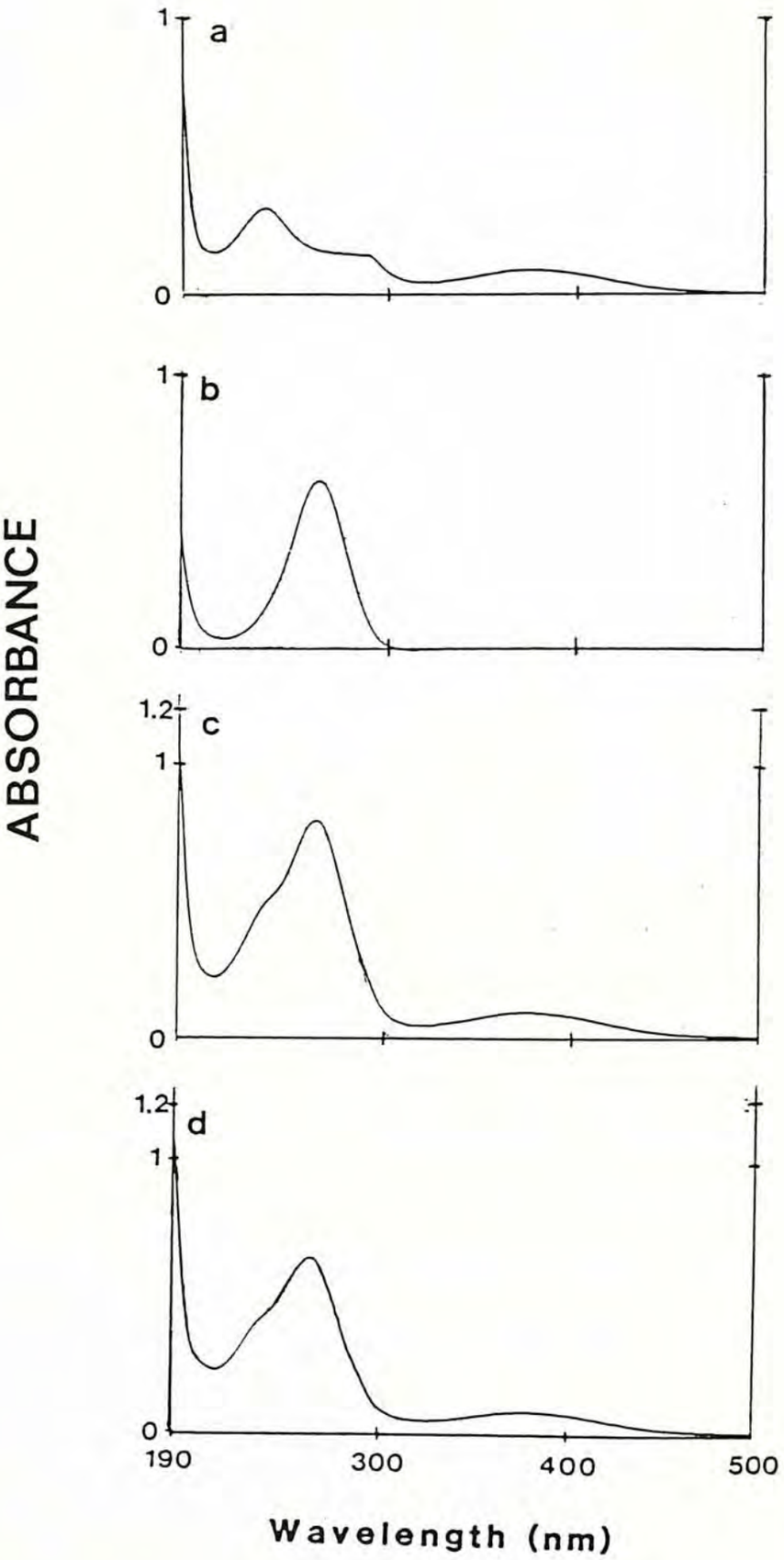
a
b
c
d
1
0
1.2
1
0
1.2
1
0
190
300
400
500
ABSORBANCE
Wavelength (nm)

Fig. 4-5. Chemical interaction of gossypol with vitamin C. The spectrophotometric absorbance profiles are: a) gossypol, b) vitamin C, c) gossypol+vitamin C (immediately), d) gossypol+vitamin C (after 10 hours). The method for the solution preparation and determination was described in Materials and Methods. Parameter used: the speed was fast and the scale was 40 nm/cm.

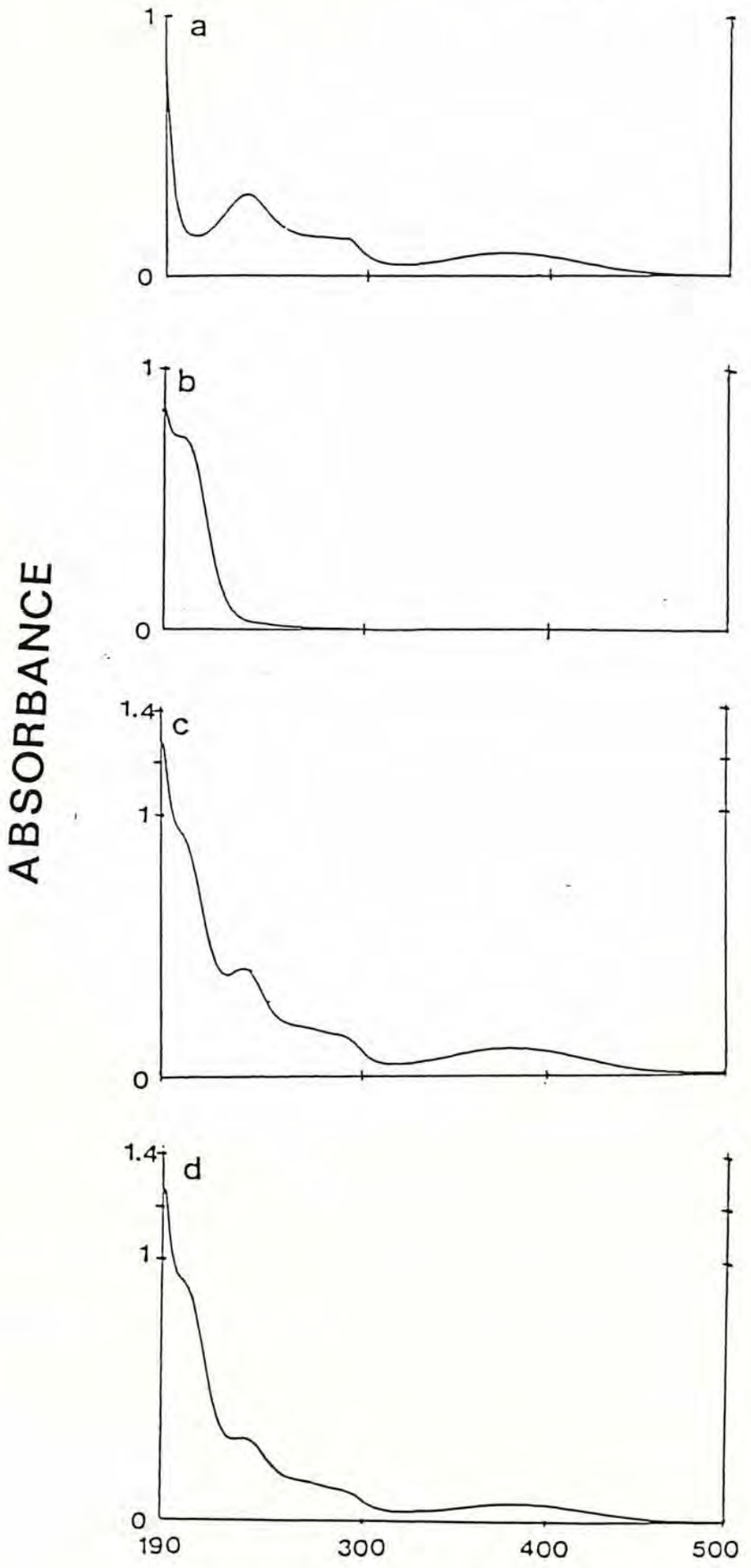
a
b
c
d
1
0
1.4
ABSORBANCE
190
300
400
500
Wavelength (nm)

Fig. 4-6. Chemical interaction of gossypol with sodium selenite. The profiles are: a) gossypol, b) sodium selenite, c) gossypol +sodium selenite (immediately), d) gossypol+sodium selenite (after 10 hours). The conditions and methods for determination was the same as described in Fig. 4-5.

b, c). Even though the mixture was related to stand for 10 hours, the profile was the same as that in immediate detection (Fig. 4-5d). This feature was also demonstrated between gossypol and sodium selenite (Fig. 4-6). This implicates that the recovery of sperm motility was not the result of the immediate action between gossypol and vitamin C or sodium selenite. In other words, the free radical production from gossypol metabolism might be eliminated by these scavengers.

C. The MDA Concentration of the Testis of Hamster after Gossypol and Scavengers Injection

The concentration of MDA (the product of lipid peroxidation) was compared, the gossypol sample contained the highest MDA concentration. This is obviously higher than that of the control (C). A supplement of vitamin C, as shown in Fig. 4-7, did reduce the MDA level and the reduction is vitamin C concentration dependent. At 10mg vitamin C, the level was resummed to the no-drug level.

D. The Antioxidant Defense Enzymes

At the end of the four-week gossypol feeding period, only the activities of catalase and glutathione transferase were slightly decreased. Yet, it was not statistically significant. But, three of the enzyme activities were

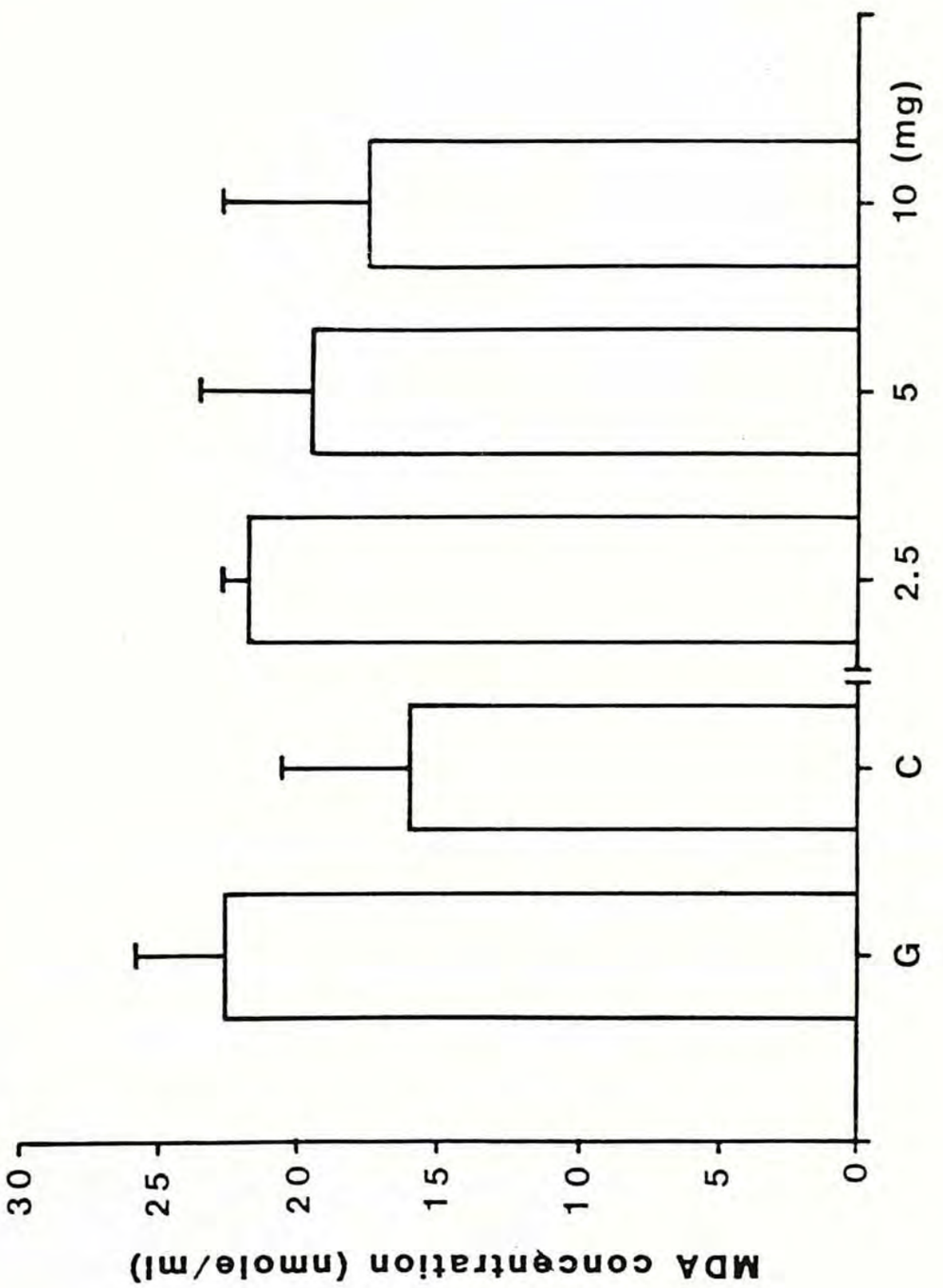
MDA concentration (nmole/ml)
0
5
10
15
20
25
30
G
C
2.5
5
10 (mg)

Fig. 4-7. The comparison of MDA concentrations in samples resulted from an injection of gossypol alone (G), oil alone (C) and that containing gossypol plus a fixed amount of vitamin C (as indicated in the horizontal axis).

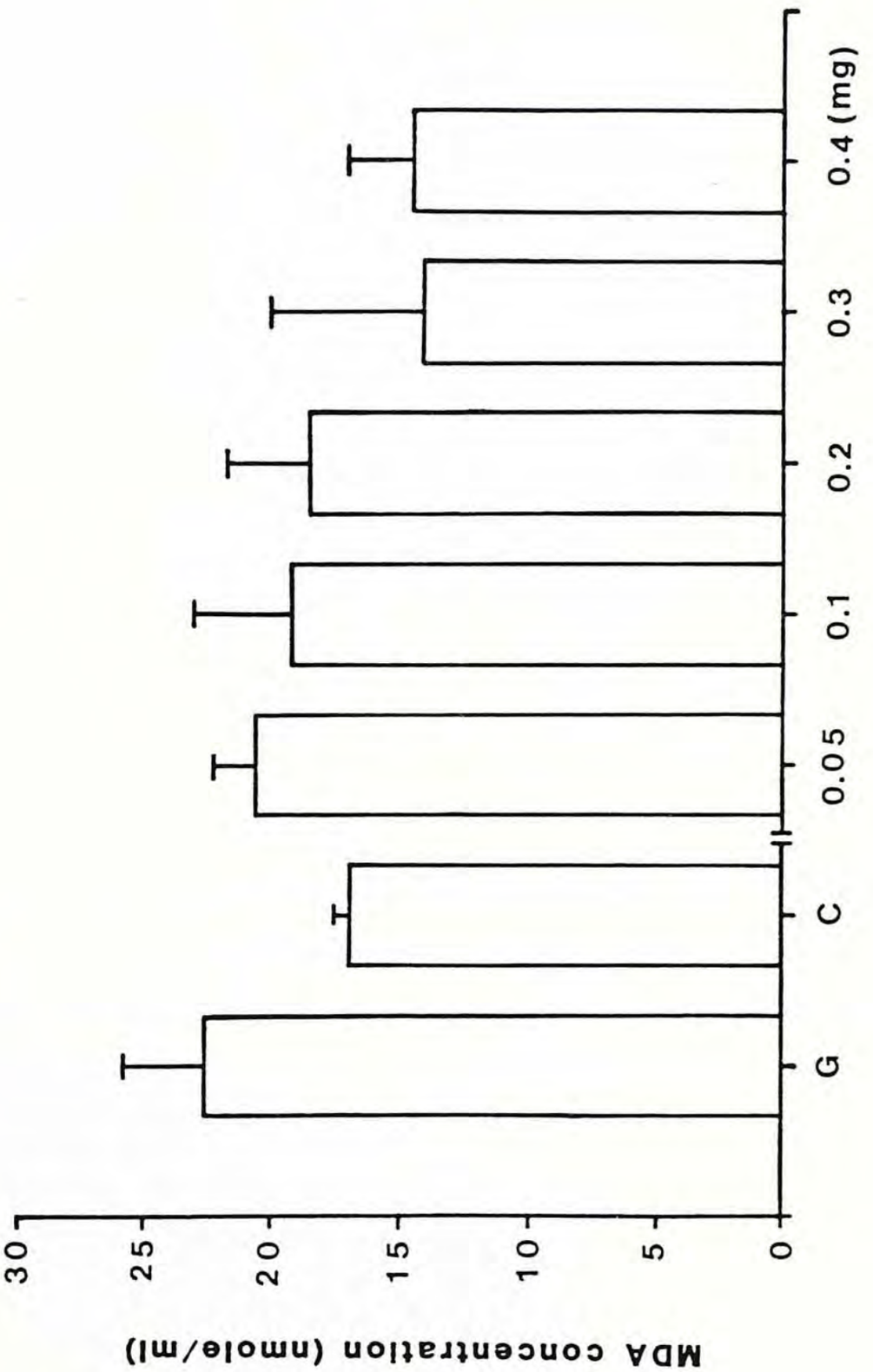
MDA concentration (nmole/ml)
0
5
10
15
20
25
30
G
C
0.05
0.1
0.2
0.3
0.4 (mg)

Fig. 4-8. The comparison of MDA concentrations in samples resulted from an injection of gossypol alone (G), oil (C) and that containing gossypol plus a fixed amount of sodium selenite (as indicated in the horizontal axis.)

slightly decreased compared with the control at the end of the fifth week after treatment which coincide with the motility profile. However, it was only statistically significant in catalase. The amounts of the four enzyme activities at the two time period were depicted in Fig. 4-9. At the same time, the sperm of all experimental hamsters were still motile at the end of the 4th week of oral gossypol treatment. However, they were decreased to less than 30% at the end of the 5th week and the colour of testis was dark when it was compared to the control.

μmoles of NADPH oxidized
/min./mg protein
a
0.08
0.04
0.02
0
4th week
5th week
μmoles of CDNB oxidized
/min./mg protein
b
4
3
2
1
0
4th week
5th week
μmoles of H2O2 oxidized
/min./mg protein
c
10
8
6
4
2
0
4th week
5th week
U/mg protein
d
180
150
120
90
60
30
0
4th week
5th week

Fig. 4-9. The effect of gossypol on the activities of antioxidant defense enzymes. The preparations were obtained from the testis of hamster. Enzyme studied: a) glutathione peroxidase. b) glutathione-S-transferase. c) catalase. d) superoxide dismutase. The empty column represents control. The shaded column represents that fed with gossypol.

IV. DISCUSSION

The mechanism of gossypol action on the male reproductive system has been postulated by various investigators. It is likely that gossypol exerts its myriad effects via several different biochemical pathways but a common underlying mechanism could also explain many of these effects. One such mechanism could be the action of free radicals.

Although, in this experiment, the majority of antioxidant defense enzymes that were examined did not show significant reduction in activity, some scavengers could eliminate the antifertility of gossypol. It seemed that gossypol did not disturb enzymatic defense system drastically. These observation was not consistent with Bender's observation (1988), who demonstrated that gossypol inhibited catalase and glutathione peroxidase in all gossypol-treated animals. The reason may be the different dosage of gossypol and the different animals used. The dose we used was 10 mg/kg/day which was the recommended dose for inducing sterility in male hamster (Chang, 1980). In this dosage, the hamsters were in good health and showed no intoxicative symptoms (a typical one was weight reduction) during treatment. But the dosage Bender (1988) used was much higher than that we used. Even the low dose (40 mg/kg/day) was four times higher than that we used although

the duration was the same. This would be highly toxic to the animals. This dosage is never used in ordinary studies. Therefore, even though Bender found that catalase and glutathione peroxidase were decreased in gossypol-treated animals, this cannot explain the effect of the smallest effective dose of gossypol. Moveover, Bender used rats for the experiment while we used hamsters. There might be species difference although we did not know the reasons.

On the other hand, among the three scavengers, only vitamin C and selenium but not vitamin E could hinder the antifertility effect of gossypol. Moreover, the MDA amount in the testis after injection of gossypol was higher than that in oil. When the concentration of vitamin C or sodium selenite was increased, the MDA amount was decreased. It seemed that gossypol could produce some reactive species of oxygen. Since the lipophilic antioxidant, vitamin E, only scavenges oxygen radicals within the membrane, it can not exhibit any protective ability in its free form and at the aqueous phase. Therefore, in this experiment, the sperm motility could not be recovered when vitamin E was injected.

Furthermore, according to the chemical structure, the poly-phenolic dialdehyde can react with oxygen to form superoxide and hydrogen peroxide during autoxidation. DePeyster *et al.* (1984) had demonstrated that gossypol

promoted the formation of reactive species of oxygen including $O_2^{\cdot -}$ and H_2O_2 in human sperm *in vitro* studies and it was also confirmed by Wu and Yu (1986) in liver and kidney microsomes *in vitro* studies. It is possible to infer that the actions of gossypol might be related to the excess production of reactive species of oxygen to overwhelm the normally efficient protective mechanisms.

However, some authors reported that gossypol brought about a stabilization of the membrane by inhibition of lipid peroxidation (Sheriff 1986; and Sheriff *et al.*, 1986). This is a contradiction in the role of gossypol in oxidative injury. Moreover, the previous demonstrations were confined to *in vitro* experiments. The situation was quite different from the *in vivo* environment. Hence, further investigations of gossypol's ability to generate oxygen radicals in more complex biological systems are warranted to determine the importance of this process in the mechanism of gossypol action.

A direct procedure for the study of free radical intermediates in biological system is electron spin resonance (e.s.r.) spectroscopy (Borg 1976 and Knowles *et al.* 1976). The technique is not very sensitive, however, and if the rate of production of free radical intermediate is low, and the lifetime short due to its high chemical reactivity, then the concentration of the free radical may

be too low for direct detection by e.s.r. In such situations it may be possible to detect and characterize the transient intermediate by spin trapping (Janzen 1971, 1980). In the future, the above two methods may be applied to study the free radical intermediates produced by gossypol. It is hoped that, from this point of view, the mechanism of gossypol antifertility action could be found very soon.

CHAPTER 5 CONCLUSIONS AND GENERAL DISCUSSION

Previous investigations have been done to clarify the action mechanism of gossypol. However, the mode of gossypol action is still not clearly understood. LDH-X, which is the specific testis enzyme, is related to the energy metabolism for sperm movement. Zinc ion is an important element which plays an important role on the maintenance of spermatogenesis. Moreover, many actions of gossypol can be explained by the production of free radicals. In order to investigate the action mechanism of gossypol, three aspects which seemed to be relating to the action mechanism of gossypol were studied.

It was observed that LDH-X activity could be detected in the testis of cock, drake, rat and mouse. Among these animals, it was documented that rat was very sensitive to gossypol antifertility, while mouse was resistant. In our experiment, the drake testis was sensitive to gossypol, while the cock testis was not. So, if LDH-X was the target enzyme of gossypol, both cock and drake testes should be sensitive to gossypol since LDH-X activity was detected in both of them. However, only drake testis, but not cock testis, was sensitive to gossypol in our experiment. On the other hand, the highest LDH-X activity was detected in mouse's testis which has been reported to be insensitive to gossypol. It was also found that LDH-X was equally susceptible to both (+)- and (-)-gossypol, and rabbit sperm

LDH-X was also inhibited by gossypol (Eliasson and Virji, 1983; Shi *et al.*, 1987). Recently, it was confirmed by other scientists that LDH-X inhibition *in vivo* was not a primary contribution to the antifertility effect produced by gossypol (Hoffer, 1985; Morris *et al.*, 1986; Lin *et al.*, 1988). Therefore, it may be concluded that LDH-X may not be the only target enzyme of gossypol.

On the other hand, our observation was in contradiction to Zinkham *et al.* who reported that there was a lack of LDH-X activities in cock and drake testes (Zinkham *et al.*, 1964). Our results showed that the LDH-X probably exists extensively in the testes of cock and drake. Recently, it is believed that using cDNA probe to detect LDH-X is a feasible and convincible way (Millan *et al.*, 1987; Edwards *et al.*, 1987). In the future, using cDNA to detect LDH-X in the animals that had been reported to contain no LDH-X activity will a more consolidating piece of work.

Besides LDH-X, zinc is supposed to be a mediator in the antifertility action of gossypol. Zinc is essential for the maintenance of spermatogenesis and it is also an important element in our body. Gossypol can easily chelate with divalent ions according to its chemical structure. If there is any change in zinc content of testes or other organs, beyond the impairment of spermatogenesis, other

side effects may occur. However, we could not observe any change in zinc content of rat's testis even though the oral dose of gossypol was up to 30 mg/kg/day for a duration of 30 days. During the experiment, the zinc content in hair and retina of the hamster and rat were also measured. The former is a well known indicator of this element whilst the latter is abundant in zinc. The zinc content in hair showed no significant difference between gossypol-treated animals and the control. But a substantial increase was observed in retina of gossypol-treated subjects. This does not necessarily imply that the antifertility effect of gossypol is developed by affecting the zinc content. This change may merely be one of the consequent side effects of gossypol on biological activities other than spermatogenesis, and may have nothing to do with antifertility.

Thirdly, the reactive species of oxygen are also another possible targets to which gossypol may act on leading to antifertility. Since the reactive species of oxygen are implicated as a cause of a multitude of pathologic changes, we investigated that whether gossypol could produce any reactive species of oxygen and/or inhibit the antioxidant defense system or not. In our studies, although gossypol could not inhibit the antioxidant defense enzymes totally, an intratesticular injection of scavengers could eliminate the effect of gossypol. Moreover, the MDA (the product of lipid peroxidation) concentration in sample

after the injection of gossypol was higher than that in control. It is decreased proportional to the concentration of vitamin C or sodium selenite. This could infer that the action mechanism of gossypol may be related to the production of oxygen free radicals. A recent *in vitro* study examining human sperm at rat liver micorsomes found that gossypol promoted the formation of reactive species of oxygen such as hydrogen peroxide and superoxide radicals (DePeyster *et al.*, 1984). It can be concluded the common mechanism that underlines all of the toxic effects of gossypol may be related to free radical injury.

Summing up all the above findings, although gossypol neither inhibits LDH-X activity nor reacts with zinc, it seems that gossypol may be related to the production of reactive free radical. It gives us a direction for future studies towards the understanding of the mechanism of gossypol action. However, an indirect method was used in this experiment. Apparently, a direct procedure such as e.s.r. spectroscopy or e.s.r. spin trapping should be taken up for the study of free radical intermediates producing from gossypol.

In the appendix, it was also found that lard could improve the effect of gossypol and enhance the absorption of gossypol. According to the chemical structure, gossypol is highly lipid-soluble. Recently, it was found that

gossypol encapsulated by liposomes crossed the blood-testis barrier more readily than free gossypol, without affecting its pharmacokinetic pattern in the circulating blood (Wang *et al.*, 1989). It is consistent with our finding. On the other hand, gossypol is highly reactive to protein (Lyman *et al.*, 1959) and it easily chelates with some divalent ions (Abou-Donia, 1976; Haas and Shirley, 1965). Since fat, protein and some divalent ions are chief ingredients in the daily food, we might expect that diet could affect the effect of gossypol. Therefore, when we consume gossypol, besides individual differences, we must consider the difference of the diet each sector of people is accustomed to. In order to obtain a maximal gossypol antifertility effect, an adjustment due to diet difference may be required.

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APPENDIX 1 THE INFLUENCE OF DIETARY LARD ON THE ANTIFERTILITY EFFECT OF GOSSYPOL

I. INTRODUCTION

The initial clinical trials of gossypol as a male antifertility agent were carried out in China in 1972 (Qian *et al.*, 1972). It was found that gossypol given orally at a dose of 60–70 mg/day for 35–42 days caused a gradual increase in the percentage of nonmotile spermatozoa in the ejaculate, followed by oligospermia, necrospermia and azoospermia in all 25 volunteers. Following the pilot clinical trial, 14 provincial and municipal districts in various parts of China joined in a concerted effort to evaluate the contraceptive effectiveness of the drug. Until 1980, the optimal dose were determined to be 20 mg/day for 60–70 days (Liu *et al.*, 1981). However, in an investigation on the antifertility effect of gossypol with a few Brazilian and Australian volunteers who were treated with gossypol 20 mg/day, it was found that 120 days, a much longer period to take effect than that for the Chinese, was required for antifertility effect (Coutinho *et al.*, 1984; Frick and Danner, 1985; Coutinho and Melo, 1988). Even in China, a few volunteers taking gossypol needed only one month to reach infertility, while most volunteers needed 2–3 months, and a small number of volunteers needed even more than 4 months (Qin *et al.*, 1979; Yu and Lu, 1980). This great variation in the initiation period of the drug

appears not to be a result of individual difference but rather an effect of other yet-to-be found external factors.

Similarly, reports on the minimal effective dose in animal models are also inconsistent. The sensitivity of rat testes toward gossypol showed marked individual variation. 5 mg/kg per day for 6 weeks (Wang *et al.*, 1972) or 4 weeks (Kalla *et al.*, 1981) led to infertility, but 6 mg/kg per day given for 5 weeks was said to be ineffective (Shi *et al.*, 1981). Using pure gossypol dissolved in salad oil, Yu (personal communication) found that the antifertility dosage for rats was 10 mg/kg daily for 5 weeks (except Sundays). But Wang *et al.* (1986) used gossypol acetic acid suspended in 1% CMC and fed the animals at 15 mg/kg/day took as long as 6 weeks (except Sundays) to show antifertility. According to the molecular weight, 15.0 mg of gossypol acetic acid is equal to 13.4 mg of pure gossypol. This means that the dosage Wang used was larger than that used by Yu, and the duration was also longer. The basic diets as a whole in these two laboratories were reported to be the same. May be the crux lies on the different vehicles.

Chemically, gossypol is a lipid soluble moiety. It is also known that gossypol can bind with protein and chelate with some divalent metal ions (Tone and Jensen, 1970; Smith and Clawson, 1970; Skutches *et al.*, 1974). Recently, Yu

(personal communication) has observed that high-protein diets were unfavourable to the effect of gossypol and also significantly reduced the amounts of both bound and free gossypol disposed in liver and testis. At the same experiment, Fe^{2+} was found to reduce the effect of gossypol on sperm motility. These indicate that the diet might influence the effect and absorption of gossypol. Since protein, fat and some divalent metal ions are common ingredients in our daily food, it is then quite possible that, besides individual variation, a change in the dietary composition may influence the effect of gossypol on its antifertility.

Since gossypol has been regarded as a promising male contraceptive for ten years, most research work was focused on the study of its antifertility action. Admittedly speaking, a lot of work has been done, even though the true target is still not in sight. However, among these reports, there is still a lack of fundamental study on the dietary effect on gossypol antifertility effectiveness. This work is crucial when comparison is needed among results obtained by various groups. Unless we have a better knowledge of this dietary effect, standardization will lag behind.

As fat as well as other oily stuff are main ingredients in our daily food, especially for those in developing countries, the presence of lard in food may

affect the solubility and hence the effective concentration of gossypol in our system. This study aims at providing such a piece of information that has been long waited for.

II. MATERIALS AND METHODS

A. Reagents

All the reagents used were of analytical grade. The following reagents were purchased from Sigma Chemical Co. (USA): pure gossypol; polyoxyethylene sorbitan mono-oleate (Tween 80). The following reagents were purchased from Riedel-de-Haen Chemical Co. (West Germany): diethylether; absolute ethanol; n-Hexane; hydrochloric acid min. 37%. Diethylether was used after distillation to make sure that it was peroxide free and used afresh. Acetic acid was purchased from Koch-light LTD (UK). Aniline was purchased from Merck Chemical Co. (West Germany) and was freshly distilled to absolute colourless. Celite was purchased from BDH Chemical LTD (UK). Lard was bought from local market, melted and stored at 4°C in the refrigerator for later use.

B. Experiment Animals

Thirty male Sprague-Dawley rats weighting about 300g were obtained from the animal house of The Chinese

University of Hong Kong. They were sexually matured and were divided into four groups. Group I and II were the experimental groups, both containing ten rats. Group III and IV were the control groups, each containing five rats. All the rats were housed in a temperature controlled (21–23°C) room with a 12 hours light/dark cycle. They were supplied with low protein diet (Laboratory Rabbit Chow) and water **ad libitum**.

C. Treatment of Animals

Pure gossypol was dissolved in lard (it was remelted at a temperature not higher than 60°C every time before applied) or suspended in 1% Tween 80. Each experimental animal was force-fed orally with a dosage of gossypol (10 mg/kg/day) for a period of ten weeks (6 days per week except Sunday). Oral feeding was administered with a plastic syringe attached to a curved, stainless steel No.14 needle with a spherical ball tip. Fresh gossypol samples were prepared weekly. It was ensured that gossypol sample were stable. The rats in the control groups were force-fed with lard or 1% Tween 80 only. All the animals were weighed once a week. Then the amount of gossypol administered was adjusted according to the body weight. All the rats were sacrificed by cervical dislocation after ten weeks of treatment.

D. Sperm Motility Observation

After the rats were sacrificed, dissection was taken on the scrotum. The testis, epididymis and vas deferens were exposed. Following the isolation of vas deferens, there was a cut at the distal part. 1ml of 0.9% sodium chloride (35°C) was added to a drop of semen approximately 0.05ml in volume for dilution. After mixing thoroughly, one drop of the diluted semen was added onto a glass slide with cover and then observed directly under low power microscope (10x10) according to WHO direct observation method (Laboratory Manual for the Examination of Human Semen-Cervical Mucur Interaction). The percentage of motile spermatozoa was determined by counting the number of motile cells per 100 spermatozoa observed in each field. Totally five fields were observed. The antifertility effects were determined by means of the presence of immotile sperm in vas deferens.

E. Gossypol Determination

It was based on Smith's method (1965) which was modified by Ko *et al.* (1979) and further modified in our laboratory. Firstly, three solutions were prepared. Solution A was prepared by adding 0.2 ml glacial acetic acid to one liter 95% ethanol. Solution B, a 60% ethanol–water solution, was prepared by diluting 715ml of

95% ethanol to one liter with distilled water and subsequently adding 200ml of ethyl ether and 0.2ml glacial acetic acid. Solution C was prepared by each liter 95% ethanol plus 200ml of ethyl ether and 0.2ml glacial acetic acid. In order to remove iron, celite was firstly washed with conc. HCl, filtered through a Buchner funnel and rinsed with distilled water for several times, dried for later use. A filter paper 40mm in diameter was placed on a Buchner funnel with suction then a suspension of 1g celite in 20ml of 95% ethanol was poured onto the filter paper for vacuum filtration. Then 1.5g celite was suspended in the homogenate and filtered through the prepared Buchner funnel. The tissue residue which would serve as the sample for bound gossypol with solution C was washed thoroughly, while the solution would serves as the sample for free gossypol. The method was based upon measuring the dianilinogossypol formed specifically through the reaction of gossypol with aniline. Suitable amount of fresh tissues were weighed to homogenize. Residue and aliquot samples were separated through the prepared Bunchner funnel. Both samples were reacted with aniline. The residue samples were extracted by hexane. They were determinated at 440 nm and calculated according to the standard curve. The experimental protocol was outlined in appendix 2.

The standard curves of free and bound gossypol were prepared by dissolving pure gossypol in 95% ethanol at

first, then followed by the above procedure.

Our method was different from Ko's and Smith's in three ways. The first difference was the reduction of the volume of solution B, distilled water and hexane consumed in bound gossypol determination. The purpose was to concentrate the amount of gossypol extracted from tissue, so that enough amount of gossypol in testis and epididymis was present for determination. The second difference was the reduction of dilution in the first step of free gossypol determination, accompanied with the transferring of 15ml aliquot instead of 10ml aliquot. The aim of this modification was the same as the first one. The last difference was the simplification of standard curve determination both in free and bound gossypol.

G. Plasma Gossypol Determination

Six male Sprague-Dawley rats were obtained from The Chinese University of Hong Kong (weighting ca 250 g). They were divided into two groups. Pure gossypol (10 mg/ml) was dissolved in corn oil and suspended in 1% Tween-80 respectively. A single oral dose (20 mg/kg body weight) of gossypol in lard or in Tween-80 was force-fed to the two groups' animals respectively. The feeding method was the same as described before. Blood was collected from the tail vein at the 2nd, 4th, 6th, 8th, 9.5th hour after treatment.

At each time interval, the collected blood samples were centrifuged at 2000g for 4 minutes at 5°C to separate red blood cells from plasma. To a test tube, 300µl plasma was added. Gossypol determination was the same as described before.

III. RESULTS

After 10 weeks of gossypol feeding, the sperm motility was 91.7 ± 1.5% in six rats and 55.6 ± 4.8% in two rats of group III (i.e. gossypol-Tween 80). However, the sperm motility was <5 ± 0.5% in four rats and was 40.2 ± 7.5% in two rats of group IV (i.e. gossypol–lard). After all, the sperm motility in group IV (i.e. gossypol-lard) was less than that in group III (i.e. gossypol-Tween-80). The sperm motility of other two groups was also >90%. It was shown in table 1. Lard could increase the amounts of both bound and free gossypol disposed in liver, testis and epididymis, especially the bound gossypol which was thought to play its role *in vivo* (Table 2). Moreover, after a single oral dose of gossypol, the plasma concentration of gossypol which was pre-dissolved in lard before feeding was higher than that suspended in Tween-80 during the whole experimental period. Following oral administration of pure gossypol, the gossypol concentration in plasma of both the gossypol-lard sample and the gossypol-Tween-80 control reached a peak level 4 hours after dosing due to similar availability of

Groups	No of rats	Carrier form	Body weight			Motility of spermatozoa(%)
			Initial	Final	%Gain	
I	5	1% Tween 80	330 ±29	464 ±51	+41	92.5 ±1.5
II	5	lard	320 ±19	458 ±24	+43	90.0 ±3.0
III	10	gossypol-tween 80	321 ±29	441 ±38	+37	73.6 ±25.5
IV	10	gossypol-lard	310 ±17	428 ±35	+38	22.6 ±20.8

Table 1. The influence of lard on sperm motility in gossypol-fed rats. 10 mg/kg/day of pure gossypol was fore-fed orally for ten weeks (6 days per week except Sunday). Sperm were obtained from vas deferens. Each value is mean ±SD. In order to see the effect of gossypol on sperm motility, one rat from either group III or IV after the 7th and 8th week of gossypol feeding was used. In group IV, two rats died during the experiment for unknown reason. After ten weeks of gossypol feeding, the immotile sperm in gossypol-Tween 80 group was less than 30%, while that in gossypol-lard group was greater than 80%.

Groups		III	IV	
Gossypol form		Gossypol-Tween 80	Gossypol-lard	ratio $\frac{\text{in lard}}{\text{in Tween 80}}$
Liver	Bound	25.5 ±5.7	33.8 ±8.6	1.3 ±0.4 *
	Free	12.2 ±4.2	13.2 ±2.2	1.0 ±0.4
Testis	Bound	5.4 ±1.5	10.5 ±2.5	1.9 ±0.7 **
	Free	4.4 ±2.2	5.9 ±2.4	1.3 ±0.8
Epididymis	Bound	5.5 ±0.9	9.3 ±3.3	1.7 ±0.6 *
	Free	5.3 ±3.5	9.6 ±7.8	1.8 ±1.9

Table 2. The distribution of gossypol on rat organs. 10mg/kg/day of pure gossypol was force-fed orally for ten weeks (6 days per week except sunday). The unit of gossypol was microgram per gram of wet tissue. The figures are expressed as mean ±SD. The result used was from the same group of animals reported in Table 1. *$p<0.05$, **$p<0.001$ compared with control.

gossypol in the alimentary tract. However, within the whole experimental period, the plasmic gossypol concentration of the gossypol-lard sample was at least 40%, at most 180% higher than that of the gossypol-Tween-80. This evidences that lard can enhance the permeability of gossypol across the barrier between intestine and the blood vessel so that the gossypol uptake can be increased, which can in turn increase the availability of gossypol in testis. The change in plasma concentration with time was presented in Fig 1. Apparently, the antifertility effect of gossypol is more effective in lard which is included in the diet.

On the other hand, there was no reduction of body weights in all rats (Table 1). They were within 428±35 g and 464±51 g. But the % gain in group III and IV (37% and 38% respectively) was less than that in group I and II (41% and 43% respectively). Since there is no significant difference in the body weight gain between the treated and the control group, it seems that within the used dose range, gossypol exhibits no toxicity. During the experiment, two rats in group IV died. However, we could not find out any reason. At the end of the 7th and 8th week, one rat was sacrificed in group III and IV for observing whether the sperm motility were decreased or not. But only after the 10th week, the number of sperm of all rats in group IV were decreased.

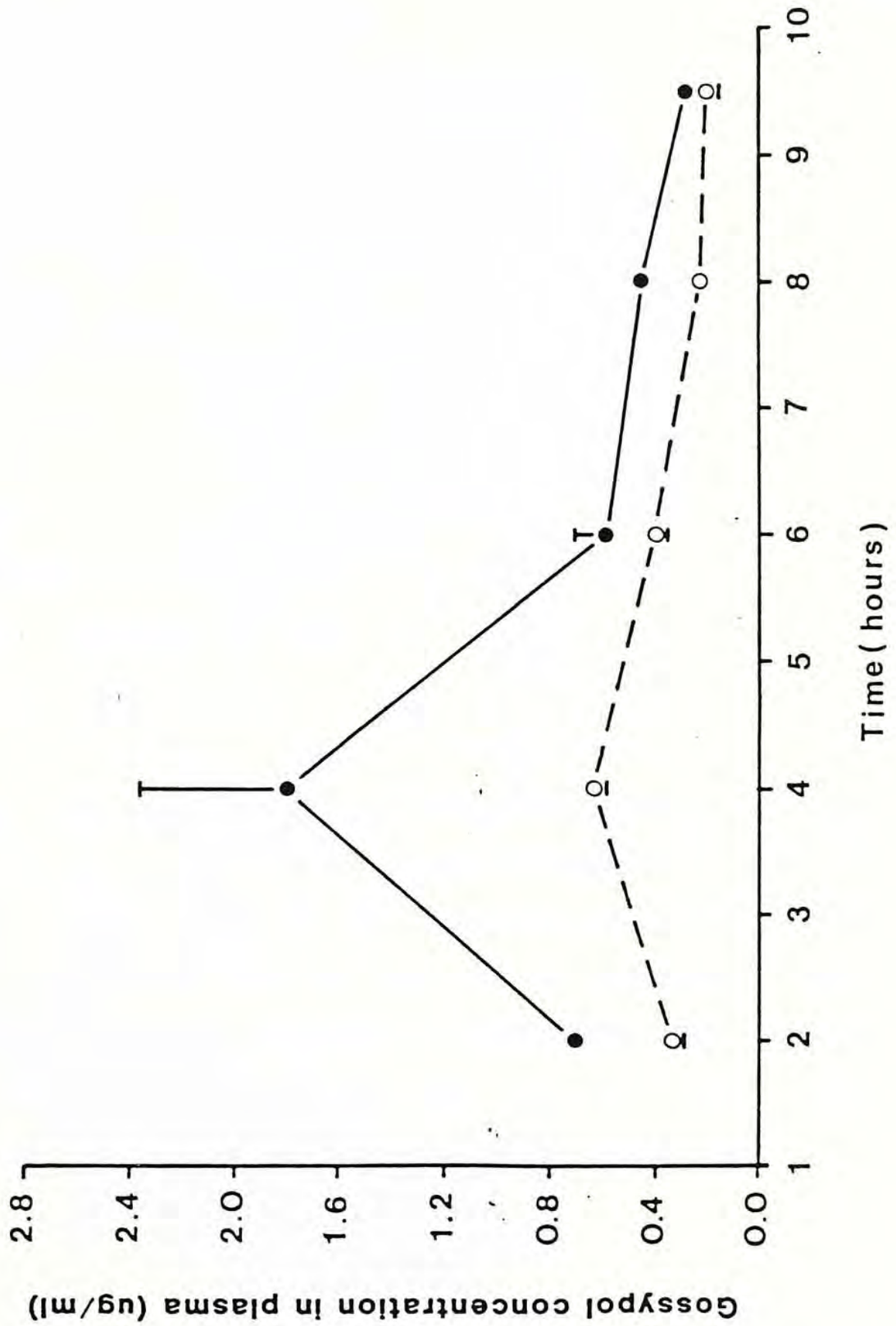
Gossypol concentration in plasma (ug/ml)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
1
2
3
4
5
6
7
8
9
10
Time (hours)

Fig. 1. The change of concentration of gossypol in plasma after the intake of the compound. A single oral dose (20 mg/kg) of pure gossypol was force-fed by gavage. Blood samples were taken from the tail vein at different interval for gossypol determination. ● represents the gossypol concentration in animal that has fed with lard, while ○ represents that in Tween-80. The values were indicated in mean±SD.

IV. DISCUSSION

There are two methyl groups and two isopropyl group in the structure of gossypol. Such groups make gossypol very lipid-soluble. Therefore, when gossypol is dissolved in organic solvent, the efficiency of emulsification and absorption of it can be increased. It was shown by the above results that lard could enhance the absorption (Fig. 1) and the deposition of gossypol (Table 2). That is probably why gossypol is more effective when it is dissolved in lard than suspended in Tween-80 suspension (Table 1). Recently, it was also found that the antispermatogenic effect of gossypol was apparently enhanced when a liposomal formulation was used (Wen *et al.*, 1981). Furthermore, gossypol encapsulated by liposomes crossed the blood-testis barrier more readily than free gossypol did, without affecting the pharmacokinetic pattern in the circulating blood (Wang *et al.*, 1989).

Owing to the chemical characteristics of gossypol, different diets and dose forms may lead to different results. That may be the reason why different reports from different laboratories concerning the antifertility of gossypol sometimes were quite different. Therefore, we should be extremely tactful when comparing these results.

By inference, gossypol would be more efficacious if

the volunteers ate more fatty food. Accordingly, we may predict that to induce antifertility in those people from area where the diet contains less fatty food, a higher dose of gossypol would be needed. In other words, perhaps the fertility could be restored in the former following cessation of gossypol, but could not in the latter. Furthermore, when we use gossypol as a "pill" for male antifertility later, we must consider the contents of diet and individual difference to adjust the dosage of gossypol used.

However, we do not recommend the oily dosage form instead of the usual tablet. It is because the latter is made of gossypol acetic acid, resulting in better stability than pure gossypol itself. Although only a very small portion of it is absorbed from the alimentary canal after administration but gossypol acetic acid is cheap and good enough as the "pill".

Theoretically the best way is to find the optimal dosage of gossypol by means of semen examination in individuals, but it is unable to do it day by day. Besides, it is also unable to unify food to all gossypol takers during administration. Therefore, it is almost impossible to define the amount and period of gossypol administration so as to solve the problem of optimum efficiency for everyone at present. These problems have to be solved

before gossypol can be practically used as a male contraceptive.

Although the dosage and chemical form we used were the same as that of Yu's (personal communication), the duration we took was longer than that taken by Yu. In comparison with the diet with which the rats were fed, both the protein and some divalent ions such as iron and zinc ion we used were higher than Yu's group used even though we used low protein diet (Laboratory Rabbit Chow.) (Table 3). Perhaps these are the main reasons why the duration we took was longer than that of Yu's. It was also another solid proof that the effect of gossypol could be affected by the normal diet, especially the fat in the diet.

Location	Protein (1%)	Zinc[c] (ppm)	Iron[c] (ppm)
Yu's group	14.5[a]	38.0	172.0
Our group	16.0[b]	112.0	266.0

Table 3. A comparison between the contents of protein, zinc and iron in diet used in two laboratories. a. Protein was determined by Kjeldahl method. b. Protein content was supplied from Purina Mills, Inc.
c. Determined by atomic absorption spectrophotometry.

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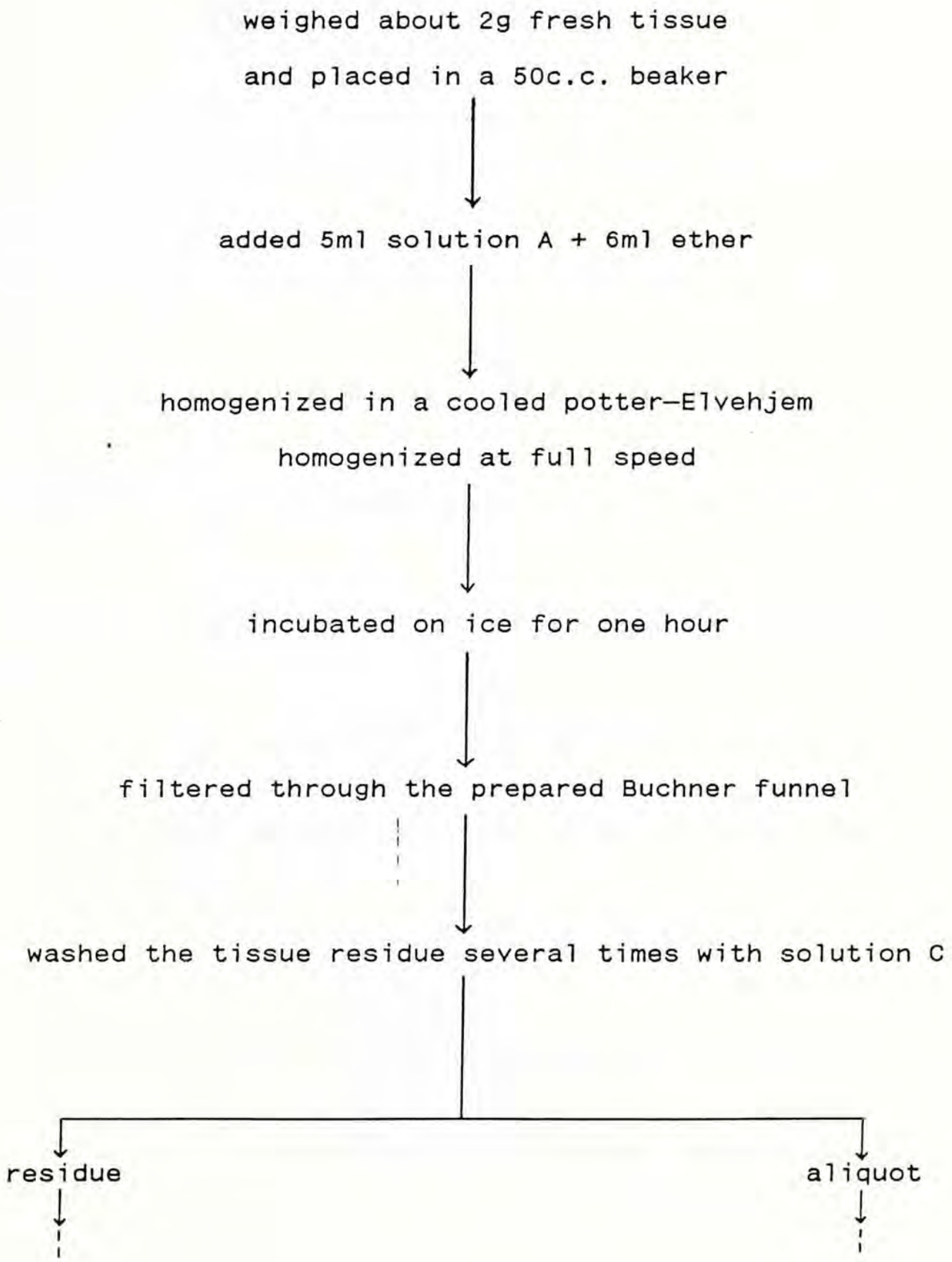
APPENDIX 2. PROTOCOL FOR GOSSYPOL DETERMINATION



residue

↓

squeezed and then transferred to a 250ml conical flask with rubber stopper

↓

added 4ml solution B and 1ml distilled water

↓

after wetted thoroughly, added 2ml aniline

↓

shook thoroughly

↓

heated on the water–bath at 60°C for 45 min. with shaking

↓

added 60–70 pieces of glass ball in 6mm diameter

↓

added 10ml hexane

↓

stoppered the flask tightly and shook vigorously for 1 hr.

↓

incubated on ice for one hour

↓

filtered with 2 pieces of Whatman No.1 filter paper

↓

took the upper layer extract for OD_{440} determination, using the hexane as a reference

↓

calculated the bounded gossypol from the standard curve

aliquot

↓

transferred to a 25ml volumetric flask

↓

diluted to the mark with solution C

↓

refiltered the solution through 2 pieces of Whatman No.1 filter paper

↓

transferred 15ml aliquot to a 25ml volumetric flask

↓

added 0.5ml aniline

↓

heated on the waterbath at 60°C for 45 min. with shaking

↓

cooled and added 1ml ether and diluted to the mark with solution C

↓

determined the absorbance at a wavelength of 440nm, using the solution C as the reference

↓

calculated the free gossypol from the standard curve